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Ny dansk patentansøgning
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Pharmaceutically active peptide conjugates having a reduced tendency towards enzymatic hydrolysis
Vor ref: 20479 DK 1

PHARMACEUTICALLY ACTIVE PEPTIDE CONJUGATES HAVING A REDUCED TENDENCY TOWARDS ENZYMATIC HYDROLYSIS

FIELD OF THE INVENTION

The present invention relates to pharmaceutically active peptide conjugates having a reduced tendency towards enzymatic cleavage.

BACKGROUND OF THE INVENTION

There exist a large number of pharmaceutically active peptides, e.g. naturally occurring in man or in animals, or synthetic analogues of such peptides. An illustrative example of such a peptide is the analgetically active peptide enkephalin which has given rise to a vast number of synthetic analogues. However, due to precisely their peptic nature, the routes of administration thereof have been rather limited. Thus, peptides are rapidly and very effectively degraded by enzymes, generally with half-lives in the range of minutes. Proteases and other proteolytic enzymes are ubiquitous, particularly in the gastro-intestinal tract, and therefore peptides are usually susceptible to degradation in multiple sites upon oral administration, and to some extent in the blood, the liver, the kidney, and the vascular endothelia. Furthermore, a given peptide is usually susceptible to degradation at more than one linkage within the backbone; each locus of hydrolysis is mediated by a certain protease.

There has been a number of attempts to protect peptides against premature degradation, such as by modification of the peptide structure, co-administration of protease inhibitors, or special formulation strategies, but they have only been met with limited success.

In the international patent application PCT/DK97/00376 (Bjarne Due Larsen and Arne Holm) prodrugs of pharmaceutically active peptides are described, wherein the pharmaceutically active peptide is coupled at its C-terminal to a peptide pre-sequence via a linker, the linker typically being an α-hydroxy carboxylic acid. These special peptide derivatives were found to have a prolonged half-life in the presence of proteolytic enzymes such as carboxypeptidase A, leucine aminopeptidase, pepsin A and α-chymotrypsin. In addition, PCT/DK97/00376 discloses (as reference compounds) four different peptides equipped with a peptide pre-sequence but without linker, namely DSIP-$(Lys\text{-}Glu)_3$, DSIP-$(Glu)_6$, Leu-enkephalin-$(Glu)_6$ and Leu-enkephalin-$(Lys)_6$.

SUMMARY OF THE INVENTION

It has now surprisingly been found that by equipping a pharmaceutically active peptide, at its C-terminal, at its N-terminal or at its C- and N-terminal, with a suitable stabilising peptide probe, it is possible to render the resulting peptide conjugate significantly less susceptible to degradation by proteases compared to the corresponding free pharmaceutically active peptide. Without being bound to any specific model for this effect, it is believed that the presence of the peptide probe induces a degree of structuring, based on hydrogen bonds, of the pharmaceutically active peptide, whereby the peptide conjugate is less susceptible to proteases in contrast to peptides in the random-coil conformation. As a result of the structuring, the peptide conjugate is much more difficult for a protease to degrade. Moreover, the resulting peptide conjugate is still pharmaceutically active, i.e. the peptide conjugate possesses the ability to exert the biological function of the free pharmaceutically active peptide.

Thus, in a first aspect the invention relates to a pharmaceutically active peptide conjugate having a reduced tendency towards enzymatic cleavage, said peptide conjugate comprises:

a pharmaceutically active peptide sequence (X) of at the most 75 amino acid units, and

a stabilising peptide probe (Z) of 2-20 amino acid units covalently bound to X at the C-terminal carbonyl function of X via a peptide bond (X-Z), or

a stabilising peptide probe (Z) of 2-20 amino acid units covalently bound to the N-terminal nitrogen atom of X via a peptide bond (Z-X), or

two stabilising peptide probes (Z), wherein each Z consists of 2-20 amino acid units, the first probe being covalently bound to X at the C-terminal carbonyl function of X via a peptide bond, the other probe being covalently bound to the N-terminal nitrogen atom of X via a peptide bond (Z-X-Z),

each amino acid unit in said stabilising peptide probe (Z) being independently selected from the group consisting of Ala, Leu, Ser, Thr, Tyr, Asn, Gln, Asp, Glu, Lys, Arg, His, Met, Orn, and amino acid units of the general formula I

$$-NH-C(R^1)(R^2)-C(=O)- \qquad (I)$$

wherein R^1 and R^2 independently are selected from hydrogen, C_{1-6}-alkyl, phenyl, and phenyl-methyl, wherein C_{1-6}-alkyl is optionally substituted with from one to three substituents selected from halogen, hydroxy, amino, cyano, nitro, sulfono, and carboxy, and phenyl and phenyl-methyl is optionally substituted with from one to three substituents selected from C_{1-6}-alkyl, C_{2-6}-alkenyl, halogen, hydroxy,

amino, cyano, nitro, sulfono, and carboxy, or R^1 and R^2 together with the carbon atom to which they are bound form a cyclopentyl, cyclohexyl, or cycloheptyl ring;

or a salt thereof, with the proviso that said pharmaceutically active peptide conjugate is not selected from
H-Trp-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu-$(Lys-Glu)_3$-OH,
H-Trp-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu-$(Glu)_6$-OH,
H-Tyr-Gly-Gly-Phe-Leu-$(Glu)_6$-OH and
H-Tyr-Gly-Gly-Phe-Leu-$(Lys)_6$-OH.

The present invention also relates to a pharmaceutical composition comprising said pharmaceutically active peptide conjugate and a pharmaceutically acceptable carrier, to a pharmaceutically active peptide conjugate for use in therapy, and to the use of a pharmaceutically active peptide conjugate for the manufacture of a pharmaceutical composition for use in therapy.

In another aspect the present invention relates to the use of a peptide conjugate, as defined herein (i.e. X-Z, Z-X, or Z-X-Z), for the manufacture of a pharmaceutical composition for the treatment or prophylaxis of a condition or disorder, where the peptide sequence X, when not bound to Z, is able to interact with a receptor (or a receptor system) involved with the condition or disorder in question, and where the interaction between X, when not bound to Z, and the receptor (or receptor system) has a therapeutic or prophylactic effect on the condition or disorder.

The present invention also relates to methods for the preparation of said pharmaceutically active peptide conjugate, to a pharmaceutically active peptide conjugate being produced by means of recombinant DNA-technology, and to the use of a stabilising peptide probe (Z) for the preparation of a pharmaceutically active peptide conjugate.

DETAILED DESCRIPTION OF THE INVENTION

In the present context, the term "amino acid unit" as used in connection with X means any naturally occurring or synthetic α, β, or γ-amino acid (whether in the L-form or the D-form) as well as side-chain modified amino acids such as modified tyrosines wherein the aromatic ring is further substituted with e.g. one or more halogens, sulfono groups, nitro groups etc., and/or the phenol group is converted into an ester group, etc, including side-chain protected amino acids, wherein the amino acid side-chains are protected in accordance with methods known to the person skilled in peptide chemistry, such as described in, e.g., M. Bodanszky and A. Bodanszky, "The Practice of Peptide Synthesis", 2. Ed, Springer-Verlag, 1994, and J. Jones, "The Chemical Synthesis of Peptides", Clarendon Press, 1991.

In the present context, the term "pharmaceutically active peptide sequence" as applied to X is intended to mean any peptide or peptide-containing structure, either naturally occurring or synthetic, having at the most 75 amino acid units (or a structure corresponding to at the most 75 amino acid units) and exerting a pharmaceutical effect in mammals such as humans.

In the present context, the pharmaceutically active peptide sequence X can be any peptide which in its native form is present as the C-terminal free carboxylic acid, such as Leu-enkephalin (H-Tyr-Gly-Gly-Phe-Leu-OH), or is present in its native form as a C-terminal amide, such as oxytocin (Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2), or is present in its native form as a C-terminal ester. Furthermore, the pharmaceutically active peptide may also contain other special structural features such as disulfide bridges as in the case insulin.

The pharmaceutically active peptide sequence X consists of at the most 75 amino acid units, such as at the most 65, 60, 55,

53, 50, 45, 40, 35, 30, 25, 20, 15, or at the most 10 amino acid units.

Peptides are utilised in a number of processes, e.g., cell-to-cell communication, some being present in the autonomic and central nervous system. Some of the latter peptides, and a number of other peptides, exert important effects on vascular and other smooth muscles. Thus, examples of interesting peptides (X) suitable for the purposes of the present invention are e.g. the vasoconstrictors angiotensin II, vasopressin, endothelin, neuropeptide Y, vasoactive intestinal peptide, substance P, neurotensin, and calcitonin, calcitonin gene-related peptide, and calcitonin gene-related peptide II. Among other pharmaceutically interesting peptides may be mentioned analgetic, antidiabetic, antibiotic, and anaesthetic peptides, etc. and, thus, the peptide may be or be reminiscent of endorphins, enkephalins, insulin, gramicidin, paracelsin, delta-sleep inducing peptide (DSIP), Gonadotropin-Releasing hormone (GnRH, sometimes referred to as Luteinizing hormone-releasing hormone, LRF or LHRH), Parathyroid 1-34 human (PTH 1-34-human), truncated EPO analogues such as those described in Science, 458-463, Vol. 273 (1996), ANF, vasotocin, bradykinin, dynorphin, endothelin, growth hormone release factor, growth hormone release peptide, oxytocin, tachykinin, ACTH, brain natriuretic polypeptide, cholecystokinin, corticotropin releasing factor, diazepam binding inhibitor fragment, FMRF-amide, galanin, gastric releasing polypeptide, gastrin, gastrin releasing peptide, glucagon, glucagon-like peptide-1, glucagon-like peptide-2, LHRH, melanin concentrating hormone, alpha-MSH, morphine modulating peptides, motilin, neurokinins, neuromedins, neuropeptide K, neuropeptide Y, PACAP, pancreatic polypeptide, peptide YY, PHM, secretin, somatostatin, substance K, substance P, TRH, vasoactive intestinal polypeptide, and such biologically active peptides as described in H.L. Lee, "Peptide and Protein Drug Delivery", Marcel Dekker Inc. 1991, Chapter 9, and references therein, Phoenix Pharmaceuticals,

Inc. "The Peptide Elite", 1997-1998 Catalogue, and Bachem, "Feinchemikalien AG", Catalog S15-1995.

It should be understood that the above-mentioned peptides as well as the sequence responsible for the biological function of these peptides can be incorporated as "X" in the peptide conjugates of the invention.

In the present context the term "free peptide" or "free peptide sequence" as applied to X, is intended to mean the pharmaceutically active peptide (or a modified or truncated analogue thereof) without the probe (or probes) Z covalently bound thereto.

The stabilising peptide probe Z may be bound to the C-terminal or the N-terminal of the pharmaceutically active peptide sequence X or two peptide probes may be bound individually to both the C- and N-terminal of X. In case the native pharmaceutically active peptide X possess a free C-terminal carboxylic acid (as in the case of Leu-enkephalin), the peptide probe Z may be attached to either the C-terminal of the peptide X or to the N-terminal of the peptide X, or the C- and N-terminal of X may both be bound to each individual peptide probe Z. Whether the probe should be attached to the peptide sequence X at its C-terminal, at its N-terminal, or both, depends on the specific peptide X and the biological function that said peptide X exerts and can be easily determined by the person skilled in the art. In some cases the biological effect may depend crucially on the negative charge at the C-terminal of the pharmaceutically active peptide X. Accordingly, in such cases the biological effect of X may be obstructed by blocking the negative charge on the C-terminal of the pharmaceutically active peptide X and it may therefore be advantageous to attach the peptide probe Z to the N-terminal of the peptide X. In a similar way, in cases where the pharmaceutically active peptide X is present in its native form as a C-terminal amide (such as

oxytocin) it may be advantageous to attach the stabilising peptide probe Z to the N-terminus of the peptide X if it is believed that the amide group has an important biological function. Thus, it should be understood that any peptide sequences corresponding to pharmaceutically active peptides X having a free C-terminal carboxyl group as well as peptides corresponding to pharmaceutically active peptides X having a C-terminal amide or ester group may be used in the peptide conjugates of the invention. However, in an interesting embodiment of the invention the peptide probe Z is attached to the C-terminal of the pharmaceutically active peptide X (whether X in its native form is a free carboxylic acid, an amide or an ester).

It is well known that many biologically active peptides also exert their desired biological effect when present in a modified or truncated form. In the case of for example insulin, porcine insulin differ from human insulin by only one amino acid unit, the B30 amino acid in porcine insulin being Ala and the B30 amino acid in human insulin being Thr. Despite this difference porcine insulin has been used as an effective diabetes drug for many years. In a similar way it has been found that the essential features for activity in the heptadecapeptide Porcine gastrin I are all contained in the C-terminal tetrapeptide and that essentially all biological effects of neurotensin are associated with the C-terminal hexapeptide. Furthermore, pharmaceutically active peptides, wherein one or more amide bonds have been modified, e.g. reduced, often exhibit a similar or even enhanced biological activity; for example the $Cys^{2}\psi[CH_2NH]Tyr^{3}$ analogue of somatostatin was found to be an even more potent growth hormone releasing agent than somatostatin itself, and also the transition state analogue $Leu^{10}\psi[CH(OH)CH_2]Val^{11}$ of angiotensin has been found to show strong inhibitory effect against the aspartic acid protease Renin. Thus, the term "modified or

truncated analogue thereof" is intended to mean such peptides that is modified by changing and/or deleting one or more amino acid units in the sequence of the native peptide, including modification of the side-chain, stereochemistry, and backbone in the individual amino acid units, such as changing one or more peptide bonds (-C(=O)-NH-) into e.g. reduced forms such as (-CH(OH)-N-), ($-CH_2-N-$), and other peptide bond mimetics such as (-C(=O)-N(CH_3)-), (-C(=O)-O), (-C(=O)-CH_2-), (-CH=CH-), (-PO_2-NH-), (SO-CH_2-), (SO_2-N-), etc.

This being said, it should be understood that the peptide sequence X in question should preferably comprise at least one peptide bond (preferably at least two peptide bonds (this naturally does not apply for a dipeptide)) susceptible to enzymatic degradation in order to fully take advantage of the present invention.

In the present context, the term "C_{1-6}-alkyl" used alone or as part of another group designates a straight, branched or cyclic saturated hydrocarbon group having from one to six carbon atoms, such as methyl, ethyl, n-propyl, isopropyl, n-butyl, isobutyl, sec.butyl, tert.butyl, n-pentyl, n-hexyl, cyclohexyl, etc.

In the present context, the term "C_{2-6}-alkenyl" designates a hydrocarbon group having from two to six carbon atoms, which may be straight, branched, or cyclic and may contain one or more double bonds, such as vinyl, allyl, 1-butenyl, 2-butenyl, isobutenyl, 1-pentenyl, 2-pentenyl, 4-pentenyl, 3-methyl-1-butenyl, 2-hexenyl, 5-hexenyl, cyclohexenyl, 2,3-dimethyl-2-butenyl etc., which may have *cis* and/or *trans* configuration.

The term "aryl" is intended to mean an aromatic, carbocyclic group such as phenyl or naphtyl.

The term "heteroaryl" includes 5- or 6-membered aromatic monocyclic heterocyclic groups containing 1-4 heteroatoms selected from nitrogen, oxygen and sulphur, such as pyrrolyl, furyl, pyrazolyl, imidazolyl, oxazolyl, isoxazolyl, thiazolyl, isothiazolyl, oxadizolyl, oxazolyl, isoxazolyl, thiazolyl, isothiazolyl, oxadiazolyl, thiadiazolyl, triazolyl, pyridyl, and aromatic bicyclic heterocyclic groups containing 1-6 heteroatoms selected from nitrogen, oxygen and sulphur, such as quinolinyl.

The term "halogen" includes fluorine, chlorine, bromine, and iodine.

The peptide probe Z is the part of the peptide conjugate responsible for introduction and/or stabilisation of a certain secondary structure into the molecule which will render the compound more stable towards degradation by proteases. It is believed that Z needs to include at least 2 amino acid units (preferably at least 3 amino acid units) in order to introduce such a stabilising structural element. On the other hand it is also believed that a sequence of more than around 20 amino acid units will not improved the stability further. Thus, Z is typically a peptide sequence of 2-20 amino acid units, e.g. in the range of 3-20, preferably in the range of 3-15, more preferably in the range of 3-10 (such as in the range of 3-8), in particular in the range of 3-7 amino acid units, such as in the range of 4-7 amino acid units, e.g. of 4, 5, 6 or 7 amino acid units.

Each of the amino acid units in the peptide sequence Z are independently selected from Ala, Leu, Ser, Thr, Tyr, Asn, Gln, Asp, Glu, Lys, Arg, His, Met, Orn, and amino acids of the formula I as defined herein. Preferably, the amino acid units are selected from Ser, Thr, Tyr, Asn, Gln, Asp, Glu, Lys, Arg, His, and Met, more preferably from Glu, Lys, and Met, especially Lys. The above-mentioned amino acids may have either

D- or L-configuration, but preferably the above-mentioned amino acids have L-configuration. As the pharmaceutically active peptide sequence X usually consists exclusively of L-amino acids, it must be expected, in order to preserve a possible stabilising helix structure of the entire peptide conjugate, that a peptide probe Z consisting only or principally of L-amino acids will be advantageous compared to a peptide probe Z consisting only or principally of D-amino acids. Furthermore, it is envisaged that a peptide probe Z consisting only or principally of D-amino acids may exert toxicological effects due to the resistance of D-peptides and D-amino acids towards biodegradation.

Thus, illustrative examples of the peptide probe Z are: Lys-Lys-Lys-Lys, Xaa-Lys-Lys-Lys, Lys-Xaa-Lys-Lys, Lys-Lys-Xaa-Lys, Lys-Lys-Lys-Xaa, Xaa-Xaa-Lys-Lys, Xaa-Lys-Xaa-Lys, Xaa-Lys-Lys-Xaa, Lys-Xaa-Xaa-Lys, Lys-Xaa-Lys-Xaa, Lys-Lys-Xaa-Xaa, Xaa-Xaa-Xaa-Lys, Xaa-Xaa-Lys-Xaa, Xaa-Lys-Xaa-Xaa, Lys-Xaa-Xaa-Xaa, Xaa-Xaa-Xaa-Xaa, Lys-Lys-Lys-Lys-Lys, Xaa-Lys-Lys-Lys-Lys, Lys-Xaa-Lys-Lys-Lys, Lys-Lys-Xaa-Lys-Lys, Lys-Lys-Lys-Xaa-Lys, Lys-Lys-Lys-Lys-Xaa, Xaa-Xaa-Lys-Lys-Lys, Xaa-Lys-Xaa-Lys-Lys, Xaa-Lys-Lys-Xaa-Lys, Xaa-Lys-Lys-Lys-Xaa, Lys-Xaa-Xaa-Lys-Lys, Lys-Xaa-Lys-Xaa-Lys, Lys-Xaa-Lys-Lys-Xaa, Lys-Lys-Xaa-Xaa-Lys, Lys-Lys-Xaa-Lys-Xaa, Lys-Lys-Lys-Xaa-Xaa, Lys-Lys-Xaa-Xaa-Xaa, Lys-Xaa-Lys-Xaa-Xaa, Lys-Xaa-Xaa-Lys-Xaa, Lys-Xaa-Xaa-Xaa-Lys, Xaa-Lys-Lys-Xaa-Xaa, Xaa-Lys-Xaa-Xaa-Lys, Xaa-Xaa-Lys-Lys-Xaa, Xaa-Xaa-Lys-Xaa-Lys, Xaa-Xaa-Xaa-Lys-Lys, Lys-Xaa-Xaa-Xaa-Xaa, Xaa-Lys-Xaa-Xaa-Xaa, Xaa-Xaa-Lys-Xaa-Xaa, Xaa-Xaa-Xaa-Lys-Xaa, Xaa-Xaa-Xaa-Xaa-Lys, Xaa-Xaa-Xaa-Xaa-Xaa, Lys-Lys-Lys-Lys-Lys-Lys, Xaa-Lys-Lys-Lys-Lys-Lys, Lys-Xaa-Lys-Lys-Lys-Lys, Lys-Lys-Xaa-Lys-Lys-Lys, Lys-Lys-Lys-Xaa-Lys-Lys, Lys-Lys-Lys-Lys-Xaa-Lys, Lys-Lys-Lys-Lys-Lys-Xaa, Xaa-Xaa-Lys-Lys-Lys-Lys, Xaa-Lys-Xaa-Lys-Lys-Lys, Xaa-Lys-Lys-Xaa-Lys-Lys, Xaa-Lys-Lys-Lys-Xaa-Lys, Xaa-Lys-Lys-Lys-Lys-Xaa, Lys-Xaa-Xaa-Lys-Lys-Lys, Lys-Xaa-Lys-Xaa-Lys-Lys, Lys-Xaa-Lys-Lys-Xaa-Lys, Lys-Xaa-Lys-Lys-Lys-Xaa, Lys-Lys-Xaa-Xaa-Lys-Lys, Lys-Lys-Xaa-Lys-Xaa-Lys, Lys-Lys-

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Due to possible solubility problems the stabilising peptide probe Z should preferably have an overall charge in the range from 0 to +15, more preferably in the range from 0 to +10, e.g. from 0 to +8, in particular from 0 to +6, such as from 0 to +4, e.g. from 0 to +3, at pH 7. Without being bound by any specific theory it is envisaged that the non-negative charge at the stabilising peptide probe Z may also to some extend facilitate transportation to and over cell membranes which possess a negative potential at the extracellular site. Thus, in order to secure a non-negative overall charge on the stabilising peptide probe Z, the peptide probe Z preferably comprises at least one Lys amino acid unit, more preferably at least two Lys amino acid units, such as at least three Lys amino acid units, e.g.

at least four Lys amino acid units, even more preferably at least five Lys amino acid units, such as at least six Lys amino acid units.

As indicated above, the amino acid units of Z may of course all be different or all be identical. However, in interesting embodiments of the present invention, the amino acid units in Z are selected from two or three different amino acids, or are identical amino acids. Examples of suitable peptide probes, wherein the amino acid units in Z are identical are e.g. $(Lys)_n$, wherein n is an integer in the range from 3 to 15, preferably in the range from 3 to 10, such as in the range from 3 to 8, e.g. in the range from 3 to 6, more preferably in the range from 4 to 6, e.g. Lys_4, Lys_5 or Lys_6. Examples of suitable peptide probes, wherein the amino acid units in Z are selected from two different amino acids are e.g. $(Lys\text{-}Xaa)_m$ or $(Xaa\text{-}Lys)_m$, wherein m is an integer in the range from 2 to 7, preferably in the range from 2 to 5, such as in the range from 2 to 4, e.g. 3, and Xaa is independently selected from the group consisting of Ser, Thr, Tyr, Asn, Gln, Asp, Glu, Arg, His and Met. More preferably such peptide probes are e.g. $(Lys\text{-}Xaa)_3$ or $(Xaa\text{-}Lys)_3$, wherein Xaa is as defined above, such as $(Lys\text{-}Glu)_3$ or $(Glu\text{-}Lys)_3$. Other examples of suitable peptide probes, wherein the amino acid units in Z are selected from two amino acid units are e.g. $Lys_p\text{-}Xaa_q$ or $Xaa_p\text{-}Lys_q$, wherein p and q are integers in the range from 1 to 14, with the proviso that p+q is in the range from 3 to 15, preferably in the range from 3 to 10, such as in the range from 3 to 8, e.g. in the range from 3 to 6, more preferably in the range from 4 to 6, e.g. 4, 5 or 6, and Xaa is independently selected from the group consisting of Ser, Thr, Tyr, Asn, Gln, Asp, Glu, Arg, His and Met. More preferably such peptide probes are e.g. $Lys_3\text{-}Xaa_3$ or $Xaa_3\text{-}Lys_3$, wherein Xaa is as defined above, such as $Lys_3\text{-}Glu_3$ or $Glu_3\text{-}Lys_3$

Examples of suitable peptide probes, wherein the amino acid units in Z are selected from three different amino acids are e.g. $Xaa^1-(Lys)_x-(Xaa^2)_y$, $Xaa^1-(Xaa^2)_x-(Lys)_y$, $(Lys)_x-(Xaa^2)_y-Xaa^1$, $(Xaa^1)_x-(Lys)_y-Xaa^2$, $(Lys)_x-Xaa^1-(Xaa^2)_y$, $(Xaa^1)_x-Xaa^2-(Lys)_y$, Xaa^1-Lys-Xaa^2-Lys, Xaa^1-Lys-Xaa^2-Lys-Xaa^2, Xaa^1-Lys-Xaa^2-Lys-Xaa^2-Lys, Xaa^1-Xaa^2-Lys-Xaa^2, Xaa^1-Xaa^2-Lys-Xaa^2-Lys, Xaa^1-Xaa^1-Lys-Xaa^2-Lys-Xaa^2, Lys-Xaa^2-Lys-Xaa^1, Lys-Xaa^2-Lys-Xaa^2-Xaa^1, Lys-Xaa^2-Lys-Xaa^2-Lys-Xaa^1, Xaa^2-Lys-Xaa^2-Xaa^1, Xaa^2-Lys-Xaa^2-Lys-Xaa^1, Xaa^2-Lys-Xaa^1-Lys-Xaa^2-Xaa^1, etc., wherein x and y are integers in the range from 1 to 4 with the proviso that x+y is at the most 5, and Xaa^1 and Xaa^2 is independently selected from the group consisting of Ala, Leu, Ser, Thr, Tyr, Asn, Gln, Asp, Glu, Arg, His, Met, Orn, and amino acids of the formula I as defined herein.

With respect to the peptide probe Z, it is envisaged that the specific amino acid units mentioned as constituents of the peptide sequence Z, i.e. Ala, Leu, Ser, Thr, Tyr, Asn, Gln, Asp, Glu, Lys, Arg, His, Met, Orn, and amino acid units of the formula I as defined herein, are amino acid units which, due to their sterical arrangement around the α-carbon atom, and probably also due to a specific electronic configuration, have certain preferences for participating in, or even stabilising or initiating, helix-like structures. The Chou-Fasman approach (Chou, P.Y. & Fasman, G.D. *Ann. Rev. Biochem.* **47**, 251-276 (1978)) is one attempt to quantify (empirically) the likelihood for a specific amino acid unit to be involved in an α-helix structure (expressed as the "Conformational parameter P_α"). Chou and Fasman's studies and related studies have, however, shown that amino acid units which have a low parameter P_α, may be found in α-helices, but of course not as often as amino acid units having a higher P_α. Thus, in the peptide probe Z, it is considered possible to include a small proportion of amino acid units which are not among the amino acid units selected above as constituents of Z, and still obtain the desired effect

from the peptide probe Z, in that the selected amino acid units are believed to compensate for any negative or neutral effect of such an alternative amino acid unit.

Thus, in embodiments which are within the scope of the present invention, it may be realistic to include up to 25% of amino acid units which are not among the amino acids preferred as constituents of Z. (By "25% percent" is referred to the number of amino acid units, i.e. no alternative amino acid units are allowed in di- and tripeptides, up to one alternative amino acid unit is allowed in tetra-, penta-, hexa-, and hepta-peptides, up to two alternative amino acid units are allowed in octapeptides, etc.). Such alternative amino acid units may be selected from Val, Ile, Pro, Phe, Gly, Trp, as well as N-methyl amino acid units, however, preferably not Pro, Gly and N-methyl amino acid units. Moreover, the C-terminal of Z may be in the form of the free acid, the amide, or an ester, e.g. methyl ester, ethyl ester, benzyl ester, etc., depending on the type of solid support material and cleavage conditions used in connection with the syntheses of the peptide conjugates as will be clear to the person skilled in the art. The N-terminal may be in the form of the free amine or a lactam.

It should be understood that the peptide conjugates of the invention may also be in the form of a salt thereof. Salts include pharmaceutically acceptable salts, such as acid addition salts and basic salts. Examples of acid addition salts are hydrochloride salts, sodium salts, calcium salts, potassium salts, etc.. Examples of basic salts are salts where the cation is selected from alkali metals, such as sodium and potassium, alkaline earth metals, such as calcium, and ammonium ions $^{+}N(R^3)_3(R^4)$, where R^3 and R^4 independently designates optionally substituted C_{1-6}-alkyl, optionally substituted C_{2-6}-alkenyl, optionally substituted aryl, or optionally substituted heteroaryl. Other examples of pharmaceutically acceptable salts

are, e.g., those described in "Remington's Pharmaceutical Sciences" 17. Ed. Alfonso R. Gennaro (Ed.), Mark Publishing Company, Easton, PA, U.S.A., 1985 and more recent editions, and in Encyclopedia of Pharmaceutical Technology.

As explained above, the peptide probe Z is the part of the peptide conjugate responsible for introducing of a certain structure into the molecule which will render the compound more stable towards protease-catalysed degradation. Therefore, the present invention also relates to the use of a stabilising peptide probe (Z) as defined above for the preparation of a pharmaceutically active peptide conjugate as defined above.

As mentioned previously the routes of administration of pharmaceutically active peptides have thus far been rather limited due to the fast biodegradation by proteases such as chymotrypsin, trypsin, carboxypeptidase A, pepsin, leucine aminopeptidase, etc. Thus, the requirements to the pharmaceutically active peptide conjugates suitable for the demanding purpose is that on the one hand, the peptide conjugate should, at least to some extend, be able to resist protease-catalysed hydrolysis, and one the other hand, the peptide conjugate should still, at least to some extend, be able to exert the desired biological effect normally provided by the free peptide X.

On this basis, *in vitro* assays have been developed which give an assessment of the capacity of a peptide conjugate to exert the desired properties. Such assays, as well as the results thereof, are illustrated in the examples. These types of assays are excellent preliminary tests which can be easily performed by the person skilled in the art to assess the suitability of any given peptide conjugate prepared according to the principles disclosed herein.

Thus, the tendency of the pharmaceutically active peptide conjugates of the invention to resist protease-catalysed hydrolysis can be measured directly by the *in vitro* enzyme assays shown in the examples. The tendency of the peptide conjugate to resist degradation can for example be expressed as a pseudo-first-order rate constant and/or as the half-life of said peptide conjugates, which can then be compared to the corresponding values of the free peptide X.

As will be apparent from the examples provided herein, it has been found that it is possible to obtain a remarkable increase in the half-life ($t_{½}$) of a pharmaceutically active peptide sequence by protecting the peptide (X) in question with a stabilising peptide probe (Z) according to the invention.

Thus, in a preferred embodiment of the invention, the ratio between the half-life of the peptide conjugate in question in the "Hydrolysis in enzyme solution test", as defined herein, and the half-life of the corresponding free peptide (X), in the "Hydrolysis in enzyme solution test", is at least 2, preferably at least 3, such as at least 5, more preferably at least 10, especially at least 20, such as at least 50, e.g. at least 100, when using one of the enzymes carboxypeptidase A, leucine aminopeptidase, α-chymotrypsin or pepsin A.

Although the proteases carboxypeptidase A, leucine aminopeptidase, α-chymotrypsin and pepsin A have been used in the tests described herein, it is envisaged that the ability of the peptide conjugates to resist protease degradation may also be tested in identical or similar test systems using other endo- or exopeptidases, such as trypsin, or mixtures of such peptidases, e.g. artificial gastric juice.

Furthermore, the ability of the peptide conjugates of the invention to exert the desired biological effect was tested in

various *in vitro* and *in vivo* assay procedures disclosed herein. Thus, preferred peptide conjugates are such conjugates which exert some biological effect, preferably a similar and in some cases even an enhanced biological effect compared to the pharmaceutically active free peptide (X).

As will be understood from examples provided herein the peptide conjugates of the invention are able to "survive" various proteolytic barriers present in the gastrointestinal environment. Thus, as demonstrated in the examples herein, it is possible to administrate a pharmaceutically active peptide conjugate (e.g. orally) as some fraction of the administered peptide conjugate (e.g. at least 1%, 2%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or even at least 99% of the total amount peptide conjugate administered) is able to enter the blood stream. Therefore, especially interesting peptide conjugates of the invention are such compounds which when administered orally in a therapeutic relevant dose (which of course depends on the actual illness or disorder to be treated and the actual peptide or peptide conjugate selected for said treatment) will be found in the blood stream in a therapeutically relevant concentration after a period of from 0.1 min to 5 hours, e.g. after a period of from 0.5 min to 3 hours, such as from 1 min to 2 hours, preferably after a period from 3 min to 1 hour, such as from 5 min to 1 hour, e.g. from 10 min to 1 hour. Therapeutically relevant concentrations of said peptide conjugates will, of course, depend on the actual illness or disorder to be treated, and such therapeutically relevant concentrations will be known to the person skilled in the art.

The above-mentioned intervals is, of course, also relevant for peptide conjugates of the invention which are administered e.g. intravenously, intraperitoneally, rectally, bucally, ocularly, pulmonary, intranasally, dermally, vaginally or intramuscularly.

Moreover, the peptide conjugates of the invention are surprisingly stable in e.g. blood serum and plasma. The suitability of the peptide conjugates of the invention for e.g. therapeutic use may be readily assessed by measuring the stability of the compounds towards degradation in human or mice blood serum or plasma. Thus, preferred peptide conjugates of the invention are such compounds which have a half-life in human or mice serum or plasma (optionally containing a buffer to secure a certain pH, e.g. a pH at 7.4) at 37°C of at least 10 minutes, such as at least 15 min, e.g. at least 0.5 h, preferably at least 1 h, such as at least 2 h, e.g. at least 3 h, more preferably at least 4 h, such as at least 5 h, e.g. at least 6 h, in particular at least 12 h, such as at least 24 h, e.g. at least 36 h.

The invention also concerns a pharmaceutical composition comprising a pharmaceutically active peptide conjugate as defined above in combination with a pharmaceutically acceptable carrier.

Such compositions may be in a form adapted to oral, parenteral (intravenous, intraperitoneal), intramuscular, rectal, intranasal, dermal, vaginal, buccal, ocularly, or pulmonary administration, preferably in a form adapted to oral administration, and such compositions may be prepared in a manner well-known to the person skilled in the art, e.g. as generally described in "Remington's Pharmaceutical Sciences", 17. Ed. Alfonso R. Gennaro (Ed.), Mark Publishing Company, Easton, PA, U.S.A., 1985 and more recent editions and in the monographs in the "Drugs and the Pharmaceutical Sciences" series, Marcel Dekker.

The invention also concerns a pharmaceutically active peptide conjugate as defined above for use in therapy, and the use of a pharmaceutically active peptide conjugate as defined above for

the manufacture of a pharmaceutical composition for use in therapy, e.g. in the treatment of disorders in the central nerveous system, in vaccine therapy, and in the treatment of HIV, cancer, diabetes, incontinence, hypertension, amnesia, Alzheimer's disease, fever, depression, sex hormone regulation, eating, schizophrenia, osteoporosis and insomnia., and as analgesics and contraceptives, and such indications known to be treated by therapy comprising administration of pharmaceutically active peptides.

As mentioned above a major obstacle to the application of peptides as clinically useful drugs is their poor delivery characteristics since most peptides are rapidly metabolised by proteolysis at most routes of administration. Consequently, a very interesting prospect of the present invention is that it is possible to prepare pharmaceutically active peptide conjugates for the treatment of mammals, such as humans, which are stabilised towards degradation by proteases and, at the same time, are able to exert a biological effect in the environment in which the free peptide (X) will exhibit a pharmaceutical action. Accordingly, the present invention also relates to the use of a pharmaceutically active peptide conjugate as defined above for the manufacture of a pharmaceutical composition for the treatment or prophylaxis of a condition or disorder, where the peptide sequence X, when not bound to Z, is able to interact with a receptor (or a receptor system) involved with the condition or disorder in question, and where the interaction between X, when not bound to Z, and the receptor (or receptor system) has a therapeutic or prophylactic effect on the condition or disorder. Thus, it should be understood that a peptide conjugate of the present invention may substitute the corresponding free peptide (X) in e.g. therapies where the free peptide X is administrated intravenous since the peptide conjugates of the invention may be administered in a more convenient way, e.g. orally, as said peptide conjugates are able to overcome proteolytic barriers

prevailing in the body. In a similar way the peptide conjugates of the invention may be used in therapies where it has not previously been possible to use the corresponding free peptide (X) as X has been readily degraded in or secreted from the body.

The peptide conjugates of the invention may be prepared by methods known per se in the art. Thus, the peptide sequences X and Z may be prepared by standard peptide-preparation techniques such as solution synthesis or Merrifield-type solid phase synthesis.

In one possible synthesis strategy, the peptide conjugates of the invention may be prepared by solid phase synthesis by first constructing the peptide probe Z using well-known standard protection, coupling and deprotection procedures, thereafter sequentially coupling the pharmaceutically active sequence X on Z in a manner similar to the construction of Z, and finally cleaving off the entire peptide conjugate from the carrier. This strategy yields a peptide conjugate, wherein the stabilising peptide probe Z is covalently bound to the pharmaceutically active peptide X at the C-terminal carbonyl function of X. If the desired peptide conjugate, however, is a peptide conjugate, wherein two stabilising probes Z are covalently and independently bound to both the C- and the N-terminal of the pharmaceutically active peptide X, the above strategy is also applicable but, as will be understood by the person skilled in the art, before cleaving the off the C-terminal bound peptide conjugate from the solid support, it is necessary to sequentially couple the second stabilising peptide probe Z to the N-terminal of X in a manner similar to the procedure described above.

A possible strategy for the preparation of peptide conjugates, wherein the stabilising peptide probe Z is covalently bound to the N-terminal nitrogen atom of X is analogous with the method

described above, i.e. said peptide conjugates may be prepared by solid phase synthesis by first constructing the pharmaceutically active peptide sequence X using well-known standard protection, coupling and deprotection procedures, thereafter sequentially coupling the stabilising peptide probe Z on X in a manner similar to the construction of X, and finally cleaving off the entire peptide conjugate from the carrier.

Another possible strategy is to prepare one or both of the two sequences X and Z (or parts thereof) separately by solution synthesis, solid phase synthesis, recombinant techniques, or enzymatic synthesis, followed by coupling of the two sequences by well-known segment condensation procedures, either in solution or using solid phase techniques or a combination thereof.

Furthermore, it is envisaged that a combination of the above-mentioned strategies may be especially applicable where a modified peptide sequence, e.g. from a biologically active peptide comprising isosteric bonds such as reduced peptide bonds, is to be coupled to a peptide probe Z. In this case it may be advantageous to prepare the immobilised fragment of Z by successive coupling of amino acids, and then couple a complete biologically active peptide sequence X (prepared in solution or fully or partially using solid phase techniques or by means of recombinant techniques) to the fragment.

Examples of suitable solid support materials (SSM) are e.g. functionalised resins such as polystyrene, polyacrylamide, polydimethylacrylamide, polyethyleneglycol, cellulose, polyethylene, polyethyleneglycol grafted on polystyrene, latex, dynabeads, etc.

It should be understood that it may be necessary or desirable that the C-terminal amino acid of the peptide probe Z or the C-

terminal amino acid of the pharmaceutically active peptide X is attached to the solid support material by means of a common linker such as 2,4-dimethoxy-4'-hydroxy-benzophenone, 4-(4-hydroxy-methyl-3-methoxyphenoxy)-butyric acid, 4-hydroxy-methylbenzoic acid, 4-hydroxymethyl-phenoxyacetic acid, 3-(4-hydroxymethylphenoxy)propionic acid, and p-[(R,S)-a[1-(9H-fluoren-9-yl)methoxyformamido]-2,4-dimethoxybenzyl]-phenoxy-acetic acid.

The peptide conjugates of the invention may be cleaved from the solid support material by means of an acid such as trifluoracetic acid, trifluoromethanesulfonic acid, hydrogenbromide, hydrogenchloride, hydrogenfluoride, etc. optionally in combination with one or more "scavengers" suitable for the purpose, e.g. ethanedithiol, triisopropylsilan, phenol, thioanisole, etc., or the peptide conjugate of the invention may be cleaved from the solid support by means of a base such as ammonia, hydrazine, an alkoxide, such as sodium ethoxide, an hydroxide, such as sodium hydroxide, etc.

Thus, the present invention also relates to a method for the preparation of a pharmaceutically active peptide conjugate, wherein Z is covalently bound to X at the C-terminal function of X (X-Z), comprising the steps of:

a) coupling an N-α-protected amino acid in the carboxyl activated form, or an N-α-protected dipeptide in the C-terminal activated form to an immobilised peptide probe H-Z-SSM, thereby forming an immobilised N-α-protected peptide fragment,

b) removing the N-α-protecting group, thereby forming an immobilised peptide fragment having an unprotected N-terminal end,

c) coupling an additional N-α-protected amino acid in the carboxyl activated form, or an additional N-α-protected dipeptide in the C-terminal activated form to the N-terminal end of the immobilised peptide fragment, and

repeating the removal/coupling step procedure in step b) and c) until the desired peptide sequence X is obtained, and then

d) cleaving off the peptide conjugate from the solid support material.

In a further aspect the present invention also relates to a method for the preparation of a pharmaceutically active peptide conjugate, wherein Z is covalently bound to the N-terminal nitrogen atom of X (Z-X), comprising the steps of:

a) coupling an N-α-protected amino acid, or an N-α-protected dipeptide to a solid support material (SSM), thereby forming an immobilised N-α-protected amino acid, or an immobilised N-α-protected dipeptide fragment,

b) removing the N-α-protecting group, thereby forming an immobilised amino acid or peptide fragment having an unprotected N-terminal end,

c) coupling an additional N-α-protected amino acid in the carboxyl activated form, or an additional N-α-protected dipeptide in the C-terminal activated form to the N-terminal end of the immobilised amino acid or peptide fragment, and

repeating the removal/coupling step procedure in step b) and c) until the desired peptide sequence X is obtained,

d) coupling an additional N-α-protected amino acid in the carboxyl activated form, or an additional N-α-protected dipeptide in the C-terminal activated form to the N-terminal end of the immobilised peptide fragment, and

repeating the removal/coupling step procedure in step b) and d) until the desired peptide sequence Z is obtained, and then

e) cleaving off the peptide conjugate from the solid support material.

In a still further aspect the present invention relates to a method for the preparation of a pharmaceutically active peptide conjugate, wherein a first probe (Z) is covalently bound to X at the C-terminal function of X and a second probe (Z) is covalently bound to the N-terminal nitrogen atom of X (Z-X-Z), comprising the steps of:

a) coupling an N-α-protected amino acid in the carboxyl activated form, or an N-α-protected dipeptide in the C-terminal activated form to an immobilised peptide probe H-Z-SSM, thereby forming an immobilised N-α-protected peptide fragment,

b) removing the N-α-protecting group, thereby forming an immobilised peptide fragment having an unprotected N-terminal end,

c) coupling an additional N-α-protected amino acid in the carboxyl activated form, or an additional N-α-protected dipeptide in the C-terminal activated form to the N-terminal end of the immobilised peptide fragment, and

repeating the removal/coupling step procedure in step b) and c) until the desired peptide sequence X is obtained, and then

d) coupling an additional N-α-protected amino acid in the carboxyl activated form, or an additional N-α-protected dipeptide in the C-terminal activated form to the N-terminal end of the immobilised peptide fragment, and

repeating the removal/coupling step procedure in step b) and d) until the desired peptide sequence Z is obtained, and then

e) cleaving off the peptide conjugate from the solid support material.

The coupling, removal and cleavage step is performed by methods known to the person skilled in the art taking into consideration the protection strategy and the selected solid phase material. In general, however, it is believed that the Boc (tert.butyloxycarbonyl) as well as the Fmoc (9-fluorenylmethyloxycarbonyl) protection strategies are applicable and that peptide bonds may be formed using the various activation procedures known to the person skilled in the art, e.g. by reacting a C-terminal activated derivative (acid halide, acid anhydride, activated ester e.g. HObt-ester, etc.) of the appropriate amino acid or peptide with the amino group of the relevant amino acid or peptide as known to a person skilled in peptide chemistry.

Furthermore, it may be necessary or desirable to include side-chain protection groups when using amino acid units carrying functional groups which are reactive under the prevailing conditions. The necessary protection scheme will be known to the person skilled in the art (see e.g. M. Bodanszky and A. Bodanszky, "The Practice of Peptide Synthesis", 2. Ed, Springer-Verlag, 1994, J. Jones, "The Chemical Synthesis of

Peptides", Clarendon Press, 1991, and A. Dryland and R.C. Sheppard (1986) J. Chem. Soc., Perkin Trans. 1, 125-137).

In another preferred embodiment of the invention, the peptide conjugates may conveniently be prepared by means of recombinant DNA-technology using general methods and principles known to the person skilled in the art, e.g. such as those described in Sambrook J, Fritsch EF, Maniatis T. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y..

The invention therefore also relates to a pharmaceutically active peptide conjugate as defined above, obtained by means of recombinant DNA-technology, e.g. by cultivating an organism which has been transformed by insertion therein of a vector comprising, in operable linkage, a peptide probe (Z) as defined herein and a pharmaceutically active peptide (X) as defined herein in either order, optionally so as to be under the control of a promoter which may be constitutive or inducible, and under conditions allowing expression of the vector, and subsequently harvesting the resulting peptide conjugate.

Examples of a suitable vector is e.g. a plasmid or a phage.

In a preferred embodiment of the invention the vector further comprises a suitable promotor.

Examples of a suitable organism is a bacterium, such as *E. coli* and *B. subtilis*, a fungus, including a yeast cell such as baker's yeast, and an eukaryotic cell derived from a higher organism, such as a plant or insect cell or a mammalian cell or cell line, such a CHO cells.

The invention is further illustrated by the following examples.

EXPERIMENTAL

Peptide synthesis

General procedures

Abbreviations used:

tBu = tert.butyl
DAMGO = Tyr-(D-Ala)-Gly-ψ[-C(=O)-N(CH_3)-]Phe-NH-CH_2-CH_2OH
DCC = dicyclohexylcarbodiimide
DCM = dichloromethane
DIC = diisopropylcarbodiimide
DIEA = N,N-diisopropylethylamine
DMAP = 4-(N,N-dimethylamino)-pyridine
Dhbt-OH = 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine
DMF = N,N-dimethylformamide
DSIP = Delta-Sleep Inducing Peptide
EDT = ethanedithiol
ES-MS = electrospray mass spectrometry
Fmoc = 9-fluorenylmethyloxycarbonyl
cHex = cyclohexyl
GLP-1 = Glucagon-Like Peptide-1
GnRH = Gonadotropin-Releasing Hormone
HMPA = 4-hydroxymethylphenoxyacetic acid
HObt = 1-hydroxybenzotriazole
HPLC = high performance liquid chromatography
NHS = N-hydroxy-succinic acid imido ester
PEG-PS = polyethyleneglycol grafted on polystyrene
Pfp = pentaflourophenyl
PTH = Parathyroid Hormone
SEM = Standard Error of Mean
TFA = trifluoroacetic acid
Z = benzyloxycarbonyl

Apparatus and synthetic strategy

Peptides were synthesized batchwise in a polyethylene vessel equipped with a polypropylene filter for filtration using 9-fluorenylmethyloxycarbonyl (Fmoc) as N-α-amino protecting group and suitable common protection groups for side-chain functionalities (A. Dryland and R.C. Sheppard (1986) J. Chem. Soc., Perkin Trans. 1, 125-137).

Solvents

Solvent DMF (*N,N*-dimethylformamide, Riedel de-Häen, Germany) was purified by passing through a column packed with a strong cation exchange resin (Lewatit S 100 MB/H strong acid, Bayer AG Leverkusen, Germany) and analysed for free amines prior to use by addition of 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (Dhbt-OH) giving rise to a yellow colour (Dhbt-O-anion) if free amines are present. Solvent DCM (dichloromethane, analytical grade, Riedel de-Häen, Germany) was used directly without purification.

Amino acids and dipeptides

Fmoc-protected amino acids were purchased from MilliGen (UK) in suitable side-chain protected forms. Otherwise protected amino acids (H-Glu(OtBu)-OtBu, H-Glu(cHex)-OH and Z-Glu(OtBu)-OH) were purchased from Bachem (Switzerland).

Coupling reagents

Coupling reagent diisopropylcarbodiimide (DIC) was purchased from (Riedel de-Häen, Germany) and distilled prior to use, dicyclohexylcarbodiimide (DCC) was purchased from Merck-Schuchardt, München, Germany, and purified by distillation.

Solid supports

Peptides synthesised according to the Fmoc-strategy were synthesised on two different types of solid support using 0.05 M or higher concentrations of Fmoc-protected activated amino acid in DMF: 1) PEG-PS (polyethyleneglycol grafted on

polystyrene; NovaSyn TG resin, 0.29 mmol/g, Novabiochem, Switzerland); 2) NovaSyn K 125 (Kieselguhr supported polydimethylacrylamide resin functionalised with sarcosine methyl ester 0.11 mmol/g; Novabiochem, Switzerland).

Catalysts and other reagents

Diisopropylethylamine (DIEA) was purchased from Aldrich, Germany, and ethylenediamine from Fluka, piperidine and pyridine from Riedel-de Häen, Frankfurt, Germany. 4-(N,N-dimethylamino)pyridine (DMAP) was purchased from Fluka, Switzerland and used as a catalyst in coupling reactions involving symmetrical anhydrides. Ethanedithiol was purchased from Riedel-de Häen, Frankfurt, Germany. 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (Dhbt-OH) and 1-hydroxybenzotriazole (HObt) were obtained from Fluka, Switzerland. FmocNHS was purchased from Aldrich, Germany. Linker (4-hydroxymethylphenoxy acetic acid (HMPA)) was obtained from Novabiochem, Switzerland and was coupled to the resin as a preformed 1-hydroxybenzotriazole (HObt) ester generated by means of DIC.

Enzymes

Carboxypeptidase A (EC 3.4.17.1) type I from Bovine Pancreas, leucine aminopeptidase (EC 3.4.11.1) type III-CP from Porcine Kidney, α-chymotrypsin (EC 4.4.21.1) from Bovine Pancreas, and pepsin A (EC 3.4.23.1) from Porcine Stomarch Mucosa Bovine Pancreas were obtained from Sigma, UK.

Coupling procedures

The first amino acid was coupled as a symmetrical anhydride in DMF generated from the appropriate N-α-protected amino acid and DIC or DCC. The following amino acids were coupled as preformed HObt esters made from appropriate N-α-protected amino acids and HObt by means of DIC in DMF. Acylations were checked by the ninhydrin test performed at 80°C in order to

prevent Fmoc deprotection during the test (Larsen, B. D. and Holm, A., Int. J. Peptide Protein Res. **43**, 1994, 1-9).

Deprotection of the N-α-amino protecting group

Deprotection of the Fmoc group was performed by treatment with 20% piperidine in DMF (1x3 and 1x7 min.), followed by wash with DMF until no yellow colour (Dhbt-O-) could be detected after addition of Dhbt-OH to the drained DMF.

Cleavage of peptide from resin with acid

Peptides were cleaved from the resins by treatment with one of the following mixtures: i) 95% trifluoroacetic acid (TFA, Riedel-de Häen, Frankfurt, Germany) and 5% water (v/v); ii) 95% TFA and 5% ethanedithiol (Aldrich) (v/v); iii) 95% TFA and 5% triisopropylsilan (Sigma) (v/v); iv) 75% TFA/20% ethanedithiol/5% water (v/v); v) 82,5% TFA/5% phenol/5% water/5% thioanisol (Aldrich)/2,5% ethandithiol (v/v) (reagent K) for 2-2.5 hours at r.t. under N_2.

The filtered resins were washed with 95% TFA-water and filtrates and washings were either 1) evaporated under reduced pressure and the residue was washed with ether and freeze dried from acetic acid-water or 2) diluted with 10% acetic acid water (v/v) and extracted with ether 3 times. Lyophilisation of the aqueous layer gave the crude peptide.

The crude freeze dried product was analysed by high-performance liquid chromatography (HPLC) and identified by electrospray ionisation mass spectrometry (ESMS).

Preformed HObt-ester

3 eq. N-α-amino protected amino acid was dissolved in DMF together with 3 eq. HObt and 3 eq DIC. The solution was left at r.t. for 10 minutes and then added to the resin, which had been

washed with a solution of 0.2% Dhbt-OH in DMF prior to the addition of the preactivated amino acid.

Preformed symmetrical anhydride

6 eq. N-α-amino protected amino acid was dissolved in DCM and cooled to 0°C. DCC (3 eq.) was added and the reaction continued for 10 min. The solvent was removed *in vacuo* and the remanence dissolved in DMF. The solution was filtered and immediately added to the resin followed by 0.1 eq. of DMAP.

Estimation of the coupling yield of the first N-α-amino protected amino acid

3-5 mg dry Fmoc-protected peptide-resin was treated with 5 ml 20% piperidine in DMF for 10 min at r.t. and the UV absorption for the dibenzofulvene-piperidine adduct was estimated at 301 nm. The yield was determined using a calculated extension coefficient ε_{301} based on a Fmoc-Ala-OH standard.

Peptide synthesis on PepSyn K resin

Dry PepSyn K (ca 500 mg), was covered by ethylenediamine and left at r.t. over night. The resin was drained and washed with DMF 10 x 15 ml, 5 min each. After draining the resin was washed with 10% DIEA in DMF v/v (2 x 15 ml, 5 min each) and finally washed with DMF until no yellow colour could be detected by addition of Dhbt-OH to the drained DMF. 3 eq. HMPA, 3 eq. HObt and 3 eq. DIC was dissolved in 10 ml DMF and left for activation for 10 min, after which the mixture was added to the resin and the coupling continued for 24 h. The resin was drained and washed with DMF (10 x 15 ml, 5 min each), and the acylation was checked by the ninhydrin test. The first amino acid was coupled as the preformed symmetrical anhydride (see above), and the coupling yields estimated as described above. It was in all cases better than 70%. The synthesis was then continued as "*batchwise* ".

Continued batchwise peptide synthesis on PepSyn K

The resin (ca. 500 mg) with the first amino acid attached was placed in a polyethylene vessel equipped with a polypropylene filter for filtration, and the Fmoc-group deprotected as described above. The remaining amino acids according to the sequence were coupled as preformed Fmoc-protected, if necessary side-chain protected, HObt esters (3 eq.) in DMF (5 ml) prepared as described above. The couplings were continued for 2 h unless otherwise specified. Excess reagent was then removed by DMF washing (12 min, flow rate 1 ml/min). All acylations were checked by the ninhydrin test performed at 80°C. After completed synthesis the peptide-resin was washed with DMF (10 min, flow rate 1 ml/min), DCM (5x5 ml, 1 min each) and finally diethyl ether (5x5 ml, 1 min each) and dried *in vacuo*.

Batchwise peptide synthesis on PEG-PS

NovaSyn TG resin (250 mg, 0.27-0.29 mmol/g) was placed in a polyethylene vessel equipped with a polypropylene filter for filtration. The resin was swelled in DMF (5 ml), and treated with 20% piperidine in DMF to secure the presence of non-protonated amino groups on the resin. The resin was drained and washed with DMF until no yellow colour could be detected after addition of Dhbt-OH to the drained DMF. HMPA (3 eq.) was coupled as a preformed HObt-ester as described above and the coupling was continued for 24 h. The resin was drained and washed with DMF (5 x 5 ml, 5 min each) and the acylation checked by the ninhydrin test. The first amino acid was coupled as a preformed symmetrical anhydride as described above. The coupling yields of the first Fmoc-protected amino acids were estimated as described above. It was in all cases better than 60%. The following amino acids according to the sequence were coupled as preformed Fmoc-protected, if necessary side-chain protected, HObt esters (3 eq.) as described above. The couplings were continued for 2 h, unless otherwise specified. The resin was drained and washed with DMF (5 x 5 ml, 5 min each) in order to remove excess reagent. All acylations were

checked by the ninhydrin test performed at 80°C. After completed synthesis the peptide-resin was washed with DMF (3x5 ml, 5 min each), DCM (3x5 ml, 1 min each) and finally diethyl ether (3x5 ml, 1 min each) and dried *in vacuo*.

HPLC conditions

Isocratic HPLC analysis was preformed on a Shimadzu system consisting of an LC-6A pump, an MERCK HITACHI L-4000 UV detector operated at 215 nm and a Rheodyne 7125 injection valve with a 2, 20, or 100 µl loop. The column used for isocratic analysis was a Spherisorb ODS-2 (100 x 3 mm; 5-µm particles). HPLC analysis using gradients was performed on a MERCK-HITACHI L-6200 Intelligent pump, an MERCK HITACHI L-4000 UV detector operated at 215 nm and a Rheodyne 7125 injection valve with a 20 µl loop. The column used was a Rescorce™ RPC 1 ml.

Buffer A was 0.1 vol% TFA in water and buffer B was 90 vol% acetonitrile, 9.9 vol% water and 0.1 vol% TFA. The Buffers were pumped through the column at a flow rate of 1.3-1.5 ml/min using the following gradient for peptide analysis 1. Linear gradient from 0% - 100% B (30 min), for enzymatic studies 2. Linear gradient from 40 - 100% B (15 min), 3. Linear gradient from 10 - 40% B (15 min), or 4. Linear gradient from 0 - 50% B (15 min). The mobile phase used for isocratic analysis will be mentioned under the description of the individual experiments.

Mass spectroscopy

Mass spectra were obtained on a Finnigan Mat LCQ instrument equipped with an electrospray (ESI) probe (ES-MS).

Peptide synthesis of individual peptides/peptide conjugates

1. Peptide synthesis of H-Tyr-Gly-Gly-Phe-Leu-Glu_6-OH (Leu-enkephalin-Glu_6-OH) on NovaSyn TentaGel

Dry NovaSyn TG resin (0.29 mmol/g, 250 mg) was placed in a polyethylene vessel equipped with a polypropylene filter for filtration and treated as described under "batchwise peptide synthesis on PEG-PS" until finishing the peptide probe Glu_6. The following amino acids forming the Leu-enkephalin sequence were coupled as preformed Fmoc-protected, if necessary side-chain protected, HObt esters (3 eq.) in DMF (5 ml) generated by means of DIC. Before each of the last five couplings the resin was washed with a solution of Dhbt-OH (80 mg in 25 ml), in order to follow the disappearance of the yellow colour as the coupling reaction proceeded. When the yellow colour was no longer visible the couplings were interrupted by washing the resin with DMF (5 x 5 ml, 5 min each). The acylations were then checked by the ninhydrin test performed at 80°C as earlier described. After completed synthesis the peptide-resin was washed with DMF (3x5 ml, 1 min each), DCM (3x5 ml, 1 min each), diethyl ether (3x5 ml, 1 min each) and dried *in vacuo*.

The peptide conjugate was cleaved from the resin as described above using 95% TFA and 5% water (v/v) as cleavage reagent and freeze dried from acetic acid. The crude freeze dried product was analysed by HPLC and found to be homogeneous without deletion and Fmoc-protected sequences. The purity was found to be better than 90% and the identity of the peptide conjugate was confirmed by ES-MS. Yield 76%.

2. Peptide synthesis of H-Tyr-Gly-Gly-Phe-Leu-Lys_6-OH (Leu-enkephalin-Lys_6-OH) on NovaSyn TentaGel.

Dry NovaSyn TG resin (0.29 mmol/g, 250 mg) was placed in a polyethylene vessel equipped with a polypropylene filter for filtration and treated as described under "batchwise peptide synthesis on PEG-PS" until finishing the peptide probe Lys_6. The following amino acids forming the Leu-enkephalin sequence were coupled as preformed Fmoc-protected HObt esters (3 eq.) in DMF (5 ml) generated by means of DIC. Before each of the last five couplings the resin was washed with a solution of Dhbt-OH

(80 mg in 25 ml), in order to follow the disappearance of the yellow colour as the coupling reaction proceed. When the yellow colour was no longer visible the couplings were interrupted by washing the resin with DMF (5 x 5 ml, 5 min each). The acylations were then checked by the ninhydrin test performed at 80°C as earlier described. After completed synthesis the peptide-resin was washed with DMF (3x5 ml, 1 min each), DCM (3x5 ml, 1 min each), diethyl ether (3x5 ml, 1 min each) and dried *in vacuo*.

The peptide conjugate was cleaved from the resin as described above using 95% TFA and 5% water (v/v) as cleavage reagent and freeze dried from acetic acid. The crude freeze dried product was analysed by HPLC and found to be homogeneous without deletion and Fmoc-protected sequences. The purity was found to be better than 98% and the identity of the peptide conjugate was confirmed by ES-MS. Yield 84%.

3. Peptide synthesis of H-Lys$_6$-Tyr-Gly-Gly-Phe-Leu-OH (H-Lys$_6$-Leu-enkephalin) on NovaSyn TentaGel.

Dry NovaSyn TG resin (0.29 mmol/g, 250 mg) was placed in a polyethylene vessel equipped with a polypropylene filter for filtration and treated as described under "batchwise peptide synthesis on PEG-PS" and the first amino acid leucine was coupled as described under coupling procedures. The following amino acids forming the H-Lys$_6$-enkephalin sequence were coupled as preformed Fmoc-protected HObt esters (3 eq.) in DMF (5 ml) generated by means of DIC and the couplings were continued for at least 2 hours. The acylations were then checked by the ninhydrin test performed at 80°C as earlier described. After completed synthesis the peptide-resin was washed with DMF (3x5 ml, 1 min each), DCM (3x5 ml, 1 min each), diethyl ether (3x5 ml, 1 min each) and dried *in vacuo*.

The peptide was cleaved from the resin as described above using 95% TFA and 5% water (v/v) as cleavage reagent and freeze dried

from acetic acid. The crude freeze dried product was analysed by HPLC and found to be homogeneous without deletion and Fmoc-protected sequences. The purity was found to be better than 98% and the identity of the peptide conjugate was confirmed by ES-MS. Yield 89%.

4. Peptide synthesis of H-Lys_6-Tyr-Gly-Gly-Phe-Leu-Lys_6-OH (H-Lys_6-Leu-enkephalin-Lys_6-OH) on NovaSyn TentaGel.

Dry NovaSyn TG resin (0.29 mmol/g, 250 mg) was placed in a polyethylene vessel equipped with a polypropylene filter for filtration and treated as described under "batchwise peptide synthesis on PEG-PS" until finishing the peptide probe Lys_6. The following amino acids forming the H-Lys_6-enkephalin sequence were coupled as preformed Fmoc-protected HObt esters (3 eq.) in DMF (5 ml) generated by means of DIC and the couplings were continued for at least 2 hours. The acylations were then checked by the ninhydrin test performed at 80°C as earlier described. After completed synthesis the peptide-resin was washed with DMF (3x5 ml, 1 min each), DCM (3x5 ml, 1 min each), diethyl ether (3x5 ml, 1 min each) and dried *in vacuo*.

The peptide was cleaved from the resin as described above using 95% TFA and 5% water (v/v) as cleavage reagent and freeze dried from acetic acid. The crude freeze dried product was analysed by HPLC and found to be homogeneous without deletion and Fmoc-protected sequences. The purity was found to be better than 98% and the identity of the peptide conjugate was confirmed by ES-MS. Yield 90%.

5. Peptide synthesis of H-Trp-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu-Glu_6-OH (DSIP-Glu_6-OH) on NovaSyn TentaGel.

Dry NovaSyn TG resin (0.29 mmol/g, 250 mg) was placed in a polyethylene vessel equipped with a polypropylene filter for filtration and treated as described under "batchwise peptide synthesis on PEG-PS" until finishing the peptide probe Glu_6. The following amino acids forming the DSIP sequence were

coupled as preformed Fmoc-protected HObt esters (3 eq.) in DMF (5 ml) generated by means of DIC. Before each of the last nine couplings the resin was washed with a solution of Dhbt-OH (80 mg in 25 ml), in order to follow the disappearance of the yellow colour as the coupling reaction proceeds. When the yellow colour was no longer visible the couplings were interrupt by washing the resin with DMF (5 x 5 ml, 5 min each). The acylations were then checked by the ninhydrin test performed at 80°C as earlier described. After completed synthesis the peptide-resin was washed with DMF (3x5 ml, 1 min each), DCM (3x5 ml, 1 min each), diethyl ether (3x5 ml, 1 min each), and dried *in vacuo*.

The peptide conjugate was cleaved from the resin as described above using 95% TFA and 5% water (v/v) as cleavage reagent and freeze dried from acetic acid. The crude freeze dried product was analysed by HPLC and found to be homogeneous without deletion and Fmoc-protected sequences. The purity was found to be better than 98% and the identity of the peptide conjugate was confirmed by ES-MS. Yield 80%.

<u>6. Peptide synthesis of H-Trp-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu-(Lys-Glu)$_3$-OH (DSIP-(Lys-Glu)$_3$-OH) on NovaSyn TentaGel.</u>

Dry NovaSyn TG resin (0.29 mmol/g, 250 mg) was placed in a polyethylene vessel equipped with a polypropylene filter for filtration and treated as described under "batchwise peptide synthesis on PEG-PS" until finishing the peptide probe (LysGlu)$_3$. The following amino acids forming the DSIP sequence were coupled as preformed Fmoc-protected HObt esters (3 eq.) in DMF (5 ml) generated by means of DIC. Before each of the last nine couplings the resin was washed with a solution of Dhbt-OH (80 mg in 25 ml), in order to follow the disappearance of the yellow colour as the coupling reaction proceeds. When the yellow colour was no longer visible the couplings were interrupt by washing the resin with DMF (5 x 5 ml, 5 min each). The acylations were then checked by the ninhydrin test

performed at 80°C as earlier described. After completed synthesis the peptide-resin was washed with DMF (3x5 ml, 1 min each), DCM (3x5 ml, 1 min each), diethyl ether (3x5 ml, 1 min each), and dried *in vacuo*.

The peptide conjugate was cleaved from the resin as described above using 95% TFA and 5% water (v/v) as cleavage reagent and freeze dried from acetic acid. The crude freeze dried product was analysed by HPLC and found to be homogeneous without deletion and Fmoc-protected sequences. The purity was found to be better than 98% and the identity of the peptide conjugate was confirmed by ES-MS. Yield 91%.

7. Peptide synthesis of H-Trp-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu-OH (DSIP) on NovaSyn TentaGel (Reference).

Dry NovaSyn TG resin (0.29 mmol/g, 250 mg) was placed in a polyethylene vessel equipped with a polypropylene filter for filtration and treated as described under "batchwise peptide synthesis on PEG-PS". The first amino acid was coupled as a preformed symmetrical anhydride as described above. The coupling yields of the first Fmoc-protected amino acids were estimated as described above. It was in all cases better than 60%. The following amino acids forming the DSIP sequence were coupled as preformed Fmoc-protected HObt esters (3 eq.) in DMF (5 ml) generated by means of DIC. Before each of the last eight couplings the resin was washed with a solution of Dhbt-OH (80 mg in 25 ml), in order to follow the disappearance of the yellow colour as the coupling reaction proceeds. When the yellow colour was no longer visible the couplings were interrupt by washing the resin with DMF (5 x 5 ml, 5 min each). The acylations were then checked by the ninhydrin test performed at 80°C as earlier described. After completed synthesis the peptide-resin was washed with DMF (3x5 ml, 1 min each), DCM (3x5 ml, 1 min each), diethyl ether (3x5 ml, 1 min each), and dried *in vacuo*.

The peptide was cleaved from the resin as described above using 95% TFA and 5% water (v/v) as cleavage reagent and freeze dried from acetic acid. The crude freeze dried product was analysed by HPLC and found to be homogeneous without deletion and Fmoc-protected sequences. The purity was found to be better than 98% and the identity of the peptide was confirmed by ES-MS. Yield 78%.

8. Peptide synthesis of H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-Lys_6-OH (Substance P-Lys_6-OH) on NovaSyn TentaGel.

Dry NovaSyn TG resin (0.29 mmol/g, 250 mg) was placed in a polyethylene vessel equipped with a polypropylene filter for filtration and treated as described under "batchwise peptide synthesis on PEG-PS" until finishing the peptide probe Lys_6. The following amino acids forming the Substance P sequence were coupled as preformed Fmoc-protected HObt esters (3 eq.) in DMF (5 ml) generated by means of DIC and the couplings were continued for at least 2 hours. The acylations were then checked by the ninhydrin test performed at 80°C as earlier described. After completed synthesis the peptide-resin was washed with DMF (3x5 ml, 1 min each), DCM (3x5 ml, 1 min each), diethyl ether (3x5 ml, 1 min each) and dried *in vacuo*.

The peptide was cleaved from the resin as described above using 95% TFA and 5% ethanedithiol as cleavage reagent and freeze dried from acetic acid. The crude freeze dried product was analysed by HPLC and found to be homogeneous without deletion and Fmoc-protected sequences. The purity was found to be better than 98% and the identity of the peptide conjugate was confirmed by ES-MS. Yield 80%.

9. Peptide synthesis of H-His-Ala-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys-Glu-Phe-Ile-Ala-Trp-Leu-Val-Lys-Gly-Arg-Lys_6-OH (GLP-1-Lys_6-OH) on NovaSyn TentaGel.

Dry NovaSyn TG resin (0.29 mmol/g, 250 mg) was placed in a polyethylene vessel equipped with a polypropylene filter for filtration and treated as described under "batchwise peptide synthesis on PEG-PS" until finishing the peptide probe Lys_6. The following amino acids forming the GLP-1 sequence were coupled as preformed Fmoc-protected HObt esters (3 eq.) in DMF (5 ml) generated by means of DIC and the couplings were continued for at least 2 hours. The acylations were then checked by the ninhydrin test performed at 80°C as earlier described. After completed synthesis the peptide-resin was washed with DMF (3x5 ml, 1 min each), DCM (3x5 ml, 1 min each), diethyl ether (3x5 ml, 1 min each) and dried *in vacuo*.

The peptide was cleaved from the resin as described above using either 95% TFA and 5% triisopropylsilan (v/v) or 75% TFA/20% ethanedithiol/5% water (v/v) as cleavage reagent and freeze dried from acetic acid. The crude freeze dried product was analysed by HPLC and it was found to contain the target peptide together with minor impurities. The crude product was purified by preparative reverse-phase HPLC and the fraction containing the GLP-1 peptide conjugate was lyophilised. The purity was found to be better than 98% and the identity of the peptide conjugate was confirmed by ES-MS. Yield 25%.

10. Peptide synthesis of pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-Lys_6-OH (GnRH-Lys_6-OH) on NovaSyn TentaGel.

Dry NovaSyn TG resin (0.29 mmol/g, 250 mg) was placed in a polyethylene vessel equipped with a polypropylene filter for filtration and treated as described under "batchwise peptide synthesis on PEG-PS" until finishing the peptide probe Lys_6. The following amino acids forming the GnRH sequence were coupled as preformed Fmoc-protected HObt esters (3 eq.) in DMF (5 ml) generated by means of DIC and the couplings were continued for at least 2 hours. The acylations were then checked by the ninhydrin test performed at 80°C as earlier described. After completed synthesis the peptide-resin was

washed with DMF (3x5 ml, 1 min each), DCM (3x5 ml, 1 min each), diethyl ether (3x5 ml, 1 min each) and dried *in vacuo*.

The peptide was cleaved from the resin as described above using either 95% TFA and 5% triisopropylsilan (v/v) or 75% TFA/20% ethanedithiol/5% water (v/v) as cleavage reagent and freeze dried from acetic acid. The crude freeze dried product was analysed by HPLC and it was found to contain the target peptide together with some impurities. The crude product was purified by preparative reverse-phase HPLC and the fraction containing the GnRH peptide conjugate was lyophilised. The purity was found to be better than 98% and the identity of the peptide conjugate was confirmed by ES-MS. Yield 37%.

11. Peptide synthesis of pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-$(Lys-Glu)_3$-OH (GnRH-$(Lys-Glu)_3$-OH) on NovaSyn TentaGel.

Dry NovaSyn TG resin (0.29 mmol/g, 250 mg) was placed in a polyethylene vessel equipped with a polypropylene filter for filtration and treated as described under "batchwise peptide synthesis on PEG-PS" until finishing the peptide probe $(Lys-Glu)_3$. The following amino acids forming the GnRH sequence were coupled as preformed Fmoc-protected HObt esters (3 eq.) in DMF (5 ml) generated by means of DIC and the couplings were continued for at least 2 hours. The acylations were then checked by the ninhydrin test performed at 80°C as earlier described. After completed synthesis the peptide-resin was washed with DMF (3x5 ml, 1 min each), DCM (3x5 ml, 1 min each), diethyl ether (3x5 ml, 1 min each) and dried *in vacuo*.

The peptide was cleaved from the resin as described above using either 95% TFA and 5% triisopropylsilan (v/v) or 75% TFA/20% ethanedithiol/5% water (v/v) as cleavage reagent and freeze dried from acetic acid. The crude freeze dried product was analysed by HPLC and it was found to contain the target peptide together with some impurities. The crude product was purified by preparative reverse-phase HPLC and the fraction containing

the GnRH peptide conjugate was lyophilised. The purity was found to be better than 98% and the identity of the peptide conjugate was confirmed by ES-MS. Yield 43%.

12. Peptide synthesis of H-Ser-Val-Ser-Glu-Ile-Gln-Leu-Met-His-Asn-Leu-Gly-Lys-His-Leu-Asn-Ser-Met-Glu-Arg-Val-Glu-Trp-Leu-Arg-Lys-Lys-Leu-Gln-Asp-Val-His-Asn-Phe-Lys_6-OH (PTH 1-34 human-Lys_6-OH) on NovaSyn TentaGel.

Dry NovaSyn TG resin (0.29 mmol/g, 250 mg) was placed in a polyethylene vessel equipped with a polypropylene filter for filtration and treated as described under "batchwise peptide synthesis on PEG-PS" until finishing the peptide probe Lys_6. The following amino acids forming the PTH sequence were coupled as preformed Fmoc-protected HObt esters (3 eq.) in DMF (5 ml) generated by means of DIC and the couplings were continued for at least 2 hours. The acylations were then checked by the ninhydrin test performed at 80°C as earlier described. After completed synthesis the peptide-resin was washed with DMF (3x5 ml, 1 min each), DCM (3x5 ml, 1 min each), diethyl ether (3x5 ml, 1 min each) and dried *in vacuo*.

The peptide was cleaved from the resin as described above using either 95% TFA and 5% triisopropylsilan (v/v) or 75% TFA/20% ethanedithiol/5% water (v/v) as cleavage reagent and freeze dried from acetic acid. The crude freeze dried product was analysed by HPLC and it was found to contain the target peptide together with impurities. The crude product was purified by preparative reverse-phase HPLC and the fraction containing the PTH peptide conjugate was lyophilised. The purity was found to be better than 98% and the identity of the peptide conjugate was confirmed by ES-MS. Yield 21%.

13. Peptide synthesis of H-Ser-Val-Ser-Glu-Ile-Gln-Leu-Met-His-Asn-Leu-Gly-Lys-His-Leu-Asn-Ser-Met-Glu-Arg-Val-Glu-Trp-Leu-Arg-Lys-Lys-Leu-Gln-Asp-Val-His-Asn-Phe-$(Lys-Glu)_3$-OH (PTH 1-34 human-$(Lys-Glu)_3$-OH) on NovaSyn TentaGel.

Dry NovaSyn TG resin (0.29 mmol/g, 250 mg) was placed in a polyethylene vessel equipped with a polypropylene filter for filtration and treated as described under "batchwise peptide synthesis on PEG-PS" until finishing the peptide probe $(Lys-Glu)_3$. The following amino acids forming the PTH sequence were coupled as preformed Fmoc-protected HObt esters (3 eq.) in DMF (5 ml) generated by means of DIC and the couplings were continued for at least 2 hours. The acylations were then checked by the ninhydrin test performed at 80°C as earlier described. After completed synthesis the peptide-resin was washed with DMF (3x5 ml, 1 min each), DCM (3x5 ml, 1 min each), diethyl ether (3x5 ml, 1 min each) and dried *in vacuo*.

The peptide was cleaved from the resin as described above using either 95% TFA and 5% triisopropylsilan (v/v) or 75% TFA/20% ethanedithiol/5% water (v/v) as cleavage reagent and freeze dried from acetic acid. The crude freeze dried product was analysed by HPLC and it was found to contain the target peptide together with impurities. The crude product was purified by preparative reverse-phase HPLC and the fraction containing the PTH peptide conjugate was lyophilised. The purity was found to be better than 98% and the identity of the peptide conjugate was confirmed by ES-MS. Yield 28%.

Hydrolysis in enzyme solution test

The decomposition of the peptide conjugate and the corresponding free pharmaceutically active peptide is studied at 37°C in a 0.05 M phosphate buffer solution. The buffer solutions contains leucine aminopeptidase (25 u/ml) at pH 7.4, carboxypeptidase A (25 u/ml) at pH 7.4, α-chymotrypsin (25 u/ml) at pH 7.4, or pepsin A (25 u/ml) at pH 2.0. The decomposition is initiated by addition of an aliquot ($\sim 10^{-7}$-10^{-8} mol) from a stock solution of the free peptide or peptide conjugate, respectively, to the test solution giving a total volume of ~5 ml reaction mixture which is kept in a water-bath

at 37°C. At appropriate time intervals samples of 50 µl are withdrawn and analysed by reversed phase HPLC as described above without previous precipitation of proteins. Half-lives of the peptide conjugate and the corresponding free peptide are determined from the slopes (i.e. k_{obs}) of the linear plots of the logarithm to the concentration of the residual derivative (HPLC peak heights) against time using the formula $t_{½}=(\ln 2)/(k_{obs})$. The ratio between the half-life of the peptide conjugate and the corresponding free peptide is calculated according to the formula:

$$ratio=(t_{½}(\text{peptide conjugate}))/(t_{½}(X)).$$

Kinetic Measurements

Hydrolysis in buffer solution.

The decomposition of some of the peptide conjugates were studied in aqueous phosphate or carbonate buffer solutions with a total buffer concentration of 0.1 - 0.05 M. In order to maintain a constant ionic strength (µ) of 0.5 a calculated amount of potassium chloride was added to the buffer solutions unless otherwise stated. The temperature was kept at 37°C during the degradation studies and pH was adjusted by adding hydrochloric acid (4M) or sodium hydroxide (2M). Hydrolysis experiments were carried out at pH 2, 7.4, and 11.

The rates of decomposition were determined by using reversed phase HPLC. The mobile phase systems used for isocratic separation were 20% acetonitrile 79.9% water 0.1% trifluoro-acetic acid or 10% acetonitrile 89.9% water 0.1% trifluoro-acetic acid. When using a linear gradient (40 - 100% B in 30 min) buffer A was 0.1% TFA in water v/v and buffer B was 90% acetonitrile 9.9% water 0.1% TFA v/v.

Hydrolysis in enzyme solution.

The decomposition of the free peptides and the peptide conjugates were studied at 37°C in a 0.05 M phosphate buffer solution containing leucine aminopeptidase (25 u/ml) at pH 7.4, carboxypeptidase A (25 u/ml) at pH 7.4, α-chymotrypsin (25 u/ml) at pH 7.4, or pepsin A (25 u/ml) at pH 2.0. The decomposition was initiated by adding an aliquot ($\sim 10^{-7}$-10^{-8} mol) from a stock solution of the peptide or peptide conjugate to the test solution giving a total volume of ~5 ml reaction mixture which was kept in a water-bath at 37°C and at appropriate intervals samples of ~50 µl were withdrawn and analysed by reversed phase HPLC as described above without previous precipitation of proteins. Pseudo-first-order rate constants for the degradations were determined from the slopes (i.e. k_{obs}) of the linear plots of the logarithm to the concentration of the residual derivative (HPLC peak heights) against time using the formula $t_{½}=(\ln 2)/(k_{obs})$. The individual assay conditions are given in the below examples.

H-Tyr-Gly-Gly-Phe-Leu-$(Glu)_6$-OH:

Hydrolysis in buffer solution

The degradation of H-Tyr-Gly-Gly-Phe-Leu-$(Glu)_6$-OH (~5 x 10^{-6} M) was studied in different aqueous buffers (0.1 M) as described above. The decomposition was followed at pH = 2, pH = 7.4 and pH = 11. The peptide conjugate was found stable at the above mentioned pH values, thus only ~5% of the peptide conjugate was degraded over a period of 24 h.

Hydrolysis in leucine aminopeptidase

The degradation of H-Tyr-Gly-Gly-Phe-Leu-$(Glu)_6$-OH ($\sim 10^{-5}$ M) in 0.05 M phosphate buffer solutions (pH = 7.4) containing leucine aminopeptidase (25 u/ml) was studied as described above. The pseudo-first-order rate constant was determined as described

earlier and was found to be 5.2 x 10^{-3} min^{-1}. The half-life was calculated to be 133 min.

Hydrolysis in carboxypeptidase A

The degradation of H-Tyr-Gly-Gly-Phe-Leu-$(Glu)_6$-OH (~10^{-5} M) in 0.05 M phosphate buffer solutions (pH = 7.4) containing carboxypeptidase A (25 u/ml) was studied as described above. The peptide conjugate was characterised as stable. Approximately 15% of the peptide conjugate was degraded over a period of 24 h.

H-Tyr-Gly-Gly-Phe-Leu-$(Lys)_6$-OH

Hydrolysis in leucine aminopetidase

The degradation of H-Tyr-Gly-Gly-Phe-Leu-$(Lys)_6$-OH (~10^{-5} M) in 0.05 M phosphate buffer solutions (pH = 7.4) containing leucine aminopeptidase (25 u/ml) was studied as described above. The pseudo-first-order rate constant was determined as described earlier and was found to be 3.6 x 10^{-3} min^{-1}. The half-life was calculated to be 191 min.

Hydrolysis in pepsin A

The degradation of H-Tyr-Gly-Gly-Phe-Leu-$(Lys)_6$-OH (~10^{-5} M) in 0.05 M phosphate buffer solutions (pH = 2.0) containing pepsin A (25 u/ml) was studied as described above. The pseudo-first-order rate constant was determined as described earlier and was found to be 1.2 x 10^{-3} min^{-1}. The half-life was calculated to be 580 min.

H-Trp-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu-$(Lys-Glu)_3$-OH

Hydrolysis in carboxypeptidase A

The degradation of H-Trp-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu-$(Lys-Glu)_3$-OH (~10^{-5} M) in 0.05 M phosphate buffer solutions (pH = 7.4) containing carboxypeptidase A 25 (u/ml) was studied as described above. The pseudo-first-order rate constant was

determined as described earlier and the half-life was calculated to be 396 min.

Hydrolysis in leucine aminopeptidase

The degradation of H-Trp-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu-(Lys-Glu)$_3$-OH (~10^{-5} M) in 0.05 M phosphate buffer solutions (pH = 7.4) containing leucine aminopeptidase (25 u/ml) was studied as described above. The pseudo-first-order rate constant was determined as described earlier and the half-life was calculated to be 145 min.

Hydrolysis in α-chymotrypsin

The degradation of H-Trp-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu-(Lys-Glu)$_3$-OH (~10^{-5} M) in 0.05 M phosphate buffer solutions (pH = 7.4) containing α-chymotrypsin (25 u/ml) was studied as described above. The pseudo-first-order rate constant was determined as described earlier and the half-life was calculated to be 613 min.

Hydrolysis in pepsin A

The degradation of H-Trp-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu-(Lys-Glu)$_3$-OH (~10^{-5} M) in 0.05 M phosphate buffer solutions (pH = 2.0) containing pepsin A (25 u/ml) was studied as described above. The peptide conjugate was characterised as stable.

H-Trp-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu-(Glu)$_6$-OH

Hydrolysis in α-chymotrypsin

The degradation of H-Trp-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu-(Glu)$_6$-OH (~10^{-5} M) in 0.05 M phosphate buffer solutions (pH = 7.4) containing α-chymotrypsin (25 u/ml) was studied as described above. The pseudo-first-order rate constant was determined as described earlier and the half-life was calculated to be 523 min.

H-Trp-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu-OH

Hydrolysis in leucine aminopeptidase

The degradation of H-Trp-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu-OH (~10^{-5} M) in 0.05 M phosphate buffer solutions (pH = 7.4) containing leucine aminopeptidase (25 u/ml) was studied as described above. The half-life was calculated to be less than 20 min.

Since native enkephalin degrades with a half-life of 10.0 minutes in aminopeptidase (20 u/ml), and with a half-life of 2.0 minutes in carboxypeptidase (1 u/ml) (see G.J. Rasmussen and H.Bundgaard, *Int. J. Pharm.*, **79**, pp 113-122 (1991)), it is concluded from the above experiments that the peptide probes of the invention provides a significant protection of a peptide sequence compared to the native peptide sequence. This is further corroborated by the results obtained for DSIP; native DSIP degrades with a half-life of less than 20 minutes in leucine aminopeptidase (25 u/ml), whereas DSIP-$(LysGlu)_3$-OH degrades with a half-life of 145 minutes under identical conditions. In general, the half-lives of DSIP derived peptide conjugates in solutions containing α-chymotrypsin or carboxypeptidase A (25 u/ml) were in the order of several hours. Although native DSIP has not been tested under these conditions it must be expected that the corresponding half-lives are significantly lower than the values obtained for the DSIP derived peptide conjugates as it is well established that native DSIP is rapidly degraded in both plasma and tissue extracts (see H.L. Lee, "Peptide and Protein Drug Delivery", Marcel Dekker Inc. 1991, Chapter 9)

***In Vitro* Studies**

μ-opioid receptor activity - 1

The affinity of the enkephalin derived peptide conjugates for the μ-opioid receptor in calf brain was determined as described by Kristensen et al. (1994) [K. Kristensen, C.B. Christensen, L.L. Christrup, and L.C. Nielsen (1994). The mu_1, mu_2, delta, kappa opioid receptor binding profiles of methadone stereoisomers and morphine. *Life Sci.* **56**, PL45-PL50.]. The activity of the peptide conjugates was determined in freshly made solutions (stored for 2 hours) and in solutions stored for 20 h at room temperature. The experimental data are summarised in Table 1.

Table 1. Inhibition of 3H-DAMGO (2 nM)

Compound	IC_{50} ± SEM (nM)	
	2 hours	20 hours
Leu-enkephalin-$(Lys)_6$-OH	14 ± 9	31 ± 4
Leu-enkephalin-$(Glu)_6$-OH	4800 ± 2700	7100 ± 1600
Leu-enkephalin-$(Lys-Glu)_3$-OH	450 ± 130	660 ± 230
Leu-enkephalin-OH (reference)	97 ± 9	56 ± 14
Met-enkephalin-OH (reference)	30	30
Naloxone (reference)	9 ± 1	4 ± 2
DAMGO (reference)	7 ± 1	8 ± 3

As shown in Table 1 all peptide conjugates were active in this binding assay, indicating that modification of the parent peptide (in this case Leu-enkephalin) by attaching a peptide probe thereto does not prevent binding to the receptor. Moreover, it can be seen that e.g. Leu-enkephalin-$(Lys)_6$-OH has an even higher affinity for the receptor than both Leu- and Met-enkephalin. In order to assess whether these binding affinities could be attributed to degradation or partial degradation of the peptide conjugates into e.g. the

corresponding free peptide, experiments were carried out after 2 and 20 h storage, respectively. As it appears from the data in Table 1, only minor effects were observed, indicating that degradation does not play any significant role for the detected affinity.

μ-opioid receptor activity - 2

The affinity of the enkephalin derived peptide conjugates as μ-opioid receptor agonists was determined using the isolated mouse vas deferens *in vitro* model described by Kramer et al. (1997) [T.H. Kramer, H. Bartosz-Bechowski, P. Davis, V.J. Hruby, and F. Porreca (1997). Extraordinary potency of a novel delta opioid receptor agonist is due in part to increased efficacy. *Life Sci.* **61:2**, 129-135.]. The activity of the peptide conjugates was determined in freshly made solutions and in solutions stored for 48 h at room temperature. The experimental data are summarised in Table 2 and 3.

Table 2. Vas Deferens activity

Peptide/peptide conjugate	Fresh	48 h
Leu-enkephalin-$(Lys)_6$-OH	aa	aa
Leu-enkephalin-$(Glu)_6$-OH	aa	aa
Leu-enkephalin-$(LysGlu)_3$-OH	a	a
Leu-enkephalin-OH (reference)	aaa	aaa

% reduction at 100 nM: a: <25%; aa: <50%; aaa: <75%

Table 3. Vas Deferens activity

Peptide/peptide conjugate	IC_{50} ± SEM (nM)
Leu-enkephalin-$(Lys)_6$-OH	140 ± 30
Leu-enkephalin-$(Glu)_6$-OH	140 ± 36
Leu-enkephalin-$(LysGlu)_3$-OH	350 ± 240
Leu-enkephalin-OH (reference)	41 ± 13

As illustrated in Table 2 and 3 the peptide conjugates of the invention inhibited the electrically induced contractions in the mouse vas deferens with IC_{50} values comparable to the IC_{50} value of Leu-enkephalin. The functional data compiled in Table 3 clearly indicate that the modified enkephalin peptide conjugates of the invention posses pharmacological properties similar to Leu-enkephalin itself.

***In Vivo* Studies**

In order to assess if Leu-enkephalin-$(Lys)_6$-OH is capable of entering the blood stream following orally, intravenous (i.v.) and intraperitoneally (i.p.) administration the following experiments were performed:

Leu-enkephalin-$(Lys)_6$-OH was administered to male mice weighing from 16 to 18 g in doses as shown in table 4. At different times the mice were sacrificed by decapitation. The total volume of blood from 4 mice was pooled and serum isolated by centrifugation. The serum concentrations of Leu-enkephalin-$(Lys)_6$-OH were determined by the displacement of 3H-DAMGO as described above. Control experiments showed that serum does not interfere with the binding assay and that IC_{50} values are independent of the presence of up to 10% serum or plasma in the assay.

Table 4. Concentration of Leu-enkephalin-$(Lys)_6$-OH in serum

admini-stration	dosage (mg/kg)	serum conc. (5 min)	serum conc. (15 min)	serum conc. (30 min)
i.v.	0.4	190 nM	170 nM	na
orally	1.6	na	190 nM	240 nM
i.p.	1.6	na	220 nM	210 nM

na: not analysed.

The data shown in Table 4 clearly indicate that some fraction of the administered peptide conjugate enters the blood stream when administered orally or i.p. Moreover, the data indicate that Leu-enkephalin-$(Lys)_6$-OH is stable in the blood for at least 30 min.

In order to evaluate the bioavailability the following experiments were carried out:

Leu-enkephalin-$(Lys)_6$-OH was administered orally to male mice weighing from 16 to 18 g in doses as shown in Table 5. The concentration of Leu-enkephalin-$(Lys)_6$-OH in serum was determined after 1 h and 4 h, respectively, using the vas deference method (as described above) and a dilution factor of 100.

Table 5. Concentration of Leu-enkephalin-$(Lys)_6$-OH in serum

admini-stration	dosage (mg/kg)	serum concentrat. (1 h)	serum concentrat. (4 h)
orally	0 (NaCl)	0	0
	5	1 μM	1 μM
	50	5 μM	1-2 μM

As illustrated in Table 5 Leu-enkephalin-$(Lys)_6$-OH is taken up in significant amounts after 1 hour, whereas most of the peptide conjugate is eliminated after 4 hours *in vivo*. Determination of the peptide conjugate concentration in serum immediately after isolation of serum and 4 hours later (i.e. *in vitro)* showed that the concentration of Leu-enkephalin-$(Lys)_6$-OH in serum has not decreased. This result indicates that enzymes present in the serum are not responsible for the degradation of the peptide conjugate.

Conclusion

Based on the results obtained it can be concluded that the stabilising peptide probe Z is of importance. Apparently, the positively charged peptide probe $(Lys)_6$ exhibited the desired effect whereas enkephalin attached to the peptide probe $(Glu)_6$ seems to be less efficient. In conclusion, the stabilising peptide probe Z is of importance for the ability of the peptide conjugates of the invention overcome the proteolytic barriers prevailing in the gastrointestinal environment. Furthermore, it is contemplated that the stabilising peptide probe may mediate the pharmaceutically active substance in crossing biological barriers such as the blood-brain-barrier, passing cell membranes, etc., and the present invention opens up the prospect of transporting peptides to the desired region by selecting an appropriate stabilising peptide probe.

CLAIMS

1. A pharmaceutically active peptide conjugate having a reduced tendency towards enzymatic cleavage, said peptide conjugate comprises:

a pharmaceutically active peptide sequence (X) of at the most 75 amino acid units, and

a stabilising peptide probe (Z) of 2-20 amino acid units covalently bound to X at the C-terminal carbonyl function of X via a peptide bond (X-Z), or

a stabilising peptide probe (Z) of 2-20 amino acid units covalently bound to the N-terminal nitrogen atom of X via a peptide bond (Z-X), or

two stabilising peptide probes (Z), wherein each Z consists of 2-20 amino acid units, the first probe being covalently bound to X at the C-terminal carbonyl function of X via a peptide bond, the other probe being covalently bound to the N-terminal nitrogen atom of X via a peptide bond (Z-X-Z),

each amino acid unit in said stabilising peptide probe (Z) being independently selected from the group consisting of Ala, Leu, Ser, Thr, Tyr, Asn, Gln, Asp, Glu, Lys, Arg, His, Met, Orn, and amino acid units of the general formula I

$$-NH-C(R^1)(R^2)-C(=O)- \qquad (I)$$

wherein R^1 and R^2 independently are selected from hydrogen, C_{1-6}-alkyl, phenyl, and phenyl-methyl, wherein C_{1-6}-alkyl is optionally substituted with from one to three substituents selected from halogen, hydroxy, amino, cyano, nitro, sulfono, and carboxy, and phenyl and phenyl-methyl is optionally substituted with from one to three substituents

selected from C_{1-6}-alkyl, C_{2-6}-alkenyl, halogen, hydroxy, amino, cyano, nitro, sulfono, and carboxy, or R^1 and R^2 together with the carbon atom to which they are bound form a cyclopentyl, cyclohexyl, or cycloheptyl ring;

or a salt thereof, with the proviso that said pharmaceutically active peptide conjugate is not selected from
H-Trp-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu-$(Lys-Glu)_3$-OH,
H-Trp-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu-$(Glu)_6$-OH,
H-Tyr-Gly-Gly-Phe-Leu-$(Glu)_6$-OH and
H-Tyr-Gly-Gly-Phe-Leu-$(Lys)_6$-OH.

2. A peptide conjugate according to claim 1, wherein Z is covalently bound to X at the C-terminal carbonyl function of X.

3. A peptide conjugate according to claim 1, wherein Z is covalently bound to the N-terminal nitrogen atom of X.

4. A peptide conjugate according to claim 1, wherein the first probe (Z) is covalently bound to X at the C-terminal carbonyl function of X and the second probe (Z) is covalently bound to the N-terminal nitrogen atom of X.

5. A peptide conjugate according to any of the preceding claims, wherein Z consists of 3-15, preferably 3-10, such as 3-8, e.g. 3-7, more preferably 4-7, such as 6 amino acid units.

6. A peptide conjugate according to any of the preceding claims, wherein the overall charge of the stabilising peptide probe (Z) is in the range from 0 to +15 at pH 7, preferably in the range from 0 to +10, such as in the range from 0 to +8, more preferably in the range from 0 to +6.

7. A peptide conjugate according to claim 5 or 6, wherein each amino acid unit in Z is independently selected from the group

consisting of Ser, Thr, Tyr, Asn, Gln, Asp, Glu, Lys, Arg, His and Met.

8. A peptide conjugate according to claim 7, wherein each amino acid unit in Z is selected from the group consisting of Glu, Lys and Met.

9. A peptide conjugate according to any of claims 5 to 8, wherein Z comprises at least one Lys amino acid unit, preferably at least two Lys amino acid units, such as at least three Lys amino acid units, e.g. at least four Lys amino acid units, more preferably at least five Lys amino acid units, such as six Lys amino acid units.

10. A peptide conjugate according to claim 9, wherein Z is $(Lys)_n$, wherein n is an integer in the range from 3 to 15, preferably in the range from 3 to 10, such as in the range from 3 to 8, e.g. in the range from 3 to 6, more preferably in the range from 4 to 6.

11. A peptide conjugate according to claim 10, wherein Z is Lys_4, Lys_5 or Lys_6.

12. A peptide conjugate according to claim 11, wherein Z is Lys_6.

13. A peptide conjugate according to any of claims 5 to 9, wherein Z is $(Lys\text{-}Xaa)_m$ or $(Xaa\text{-}Lys)_m$, wherein m is an integer in the range from 2 to 7, and each Xaa is independently selected from the group consisting of Ser, Thr, Tyr, Asn, Gln, Asp, Glu, Arg, His and Met.

14. A peptide conjugate according to claim 13, wherein Z is $(Lys\text{-}Xaa)_3$ or $(Xaa\text{-}Lys)_3$, wherein each Xaa is independently selected from the group consisting of Ser, Thr, Tyr, Asn, Gln, Asp, Glu, Arg, His and Met.

15. A peptide conjugate according to claim 14, wherein Z is $(Lys\text{-}Glu)_3$ or $(Glu\text{-}Lys)_3$.

16. A peptide conjugate according to any of claims 5 to 9, wherein Z is $Lys_p\text{-}Xaa_q$ or $Xaa_p\text{-}Lys_q$, wherein p and q are integers in the range from 1 to 14, with the proviso that p+q is in the range of 3-15, and each Xaa is independently selected from the group consisting of Ser, Thr, Tyr, Asn, Gln, Asp, Glu, Arg, His and Met.

17. A peptide conjugate according to claim 16, wherein Z is $Lys_3\text{-}Xaa_3$ or $Xaa_3\text{-}Lys_3$, wherein each Xaa is independently selected from the group consisting of Ser, Thr, Tyr, Asn, Gln, Asp, Glu, Arg, His and Met.

18. A peptide conjugate according to claim 17, wherein Z is $Lys_3\text{-}Glu_3$ or $Glu_3\text{-}Lys_3$,

19. A peptide conjugate according to any of claims 5 to 18, wherein Z consists of L-amino acids only.

20. A peptide conjugate according to any of the preceding claims, wherein said pharmaceutically active peptide sequence (X) consists of at the most 70 amino acid units, such as at the most 65, e.g. a the most 60, preferably at the most 55, such as at the most 53, e.g. at the most 50.

21. A peptide conjugate according to claim 16, wherein X is selected from the group consisting of enkephalin, angiotensin II, vasopressin, endothelin, vasoactive intestinal peptide, substance P, neurotensin, endorphins, insulin, gramicidin, paracelsin, delta-sleep inducing peptide, Gonadotropin-Releasing hormone, Parathyroid 1-34 human, truncated EPO analogues, Atrial natriuretic peptide (ANP, ANF), vasotocin, bradykinin, dynorphin, growth hormone release factor, growth

hormone release peptide, oxytocin, calcitonin, calcitonin gene-related peptide, calcitonin gene-related peptide II, growth hormone releasing peptide, tachykinin, Adrenocorticotropic hormone (ACTH), brain natriuretic polypeptide, brain natriuretic peptide, cholecystokinin, corticotropin releasing factor, diazepam binding inhibitor fragment, FMRF-amide, galanin, gastric releasing polypeptide, gastric inhibitory polypeptide gastrin, gastrin releasing peptide, glucagon, glucagon-like peptide-1, glucagon-like peptide-2, LHRH, melanin concentrating hormone, melanocyte stimulating hormone (MSH), alpha-MSH, morphine modulating peptides, motilin, neurokinin A, neurokinin B, neuromedin B, neuromedin C, neuromedin K, neuromedin N, neuromedin U, neuropeptide K, neuropeptide Y, pituitary adenylate cyclase activating polypeptide (PACAP), pancreatic polypeptide, peptide YY, peptide histidine-methionine amide (PHM), secretin, somatostatin, substance K, substance P, thyrotropin-releasing hormone (TRH), vasoactive intestinal polypeptide, kyotorphin, melanostatin (MIF-1), and any modified or truncated analogue thereof.

22. A peptide conjugate according to any of the preceding claims, wherein the ratio between the half-life of said peptide conjugate in the "Hydrolysis in enzyme solution test", as defined herein, and the half-life of the corresponding pharmaceutically active peptide sequence (X) in the "Hydrolysis in enzyme solution test", is at least 2, preferably at least 3, such as at least 5, more preferably at least 7, such as at least 9, e.g. at least 10, when using the enzyme carboxypeptidase A

23. A peptide conjugate according to any of the preceding claims, wherein the ratio between the half-life of said peptide conjugate in the "Hydrolysis in enzyme solution test", as defined herein, and the half-life of the corresponding pharmaceutically active peptide sequence (X) in the "Hydrolysis in enzyme solution test", is at least 2, preferably at least 3,

such as at least 5, more preferably at least 7, such as at least 9, e.g. at least 10, when using the enzyme leucine aminopeptidase.

24. A peptide conjugate according to any of the preceding claims, wherein the ratio between the half-life of said peptide conjugate in the "Hydrolysis in enzyme solution test", as defined herein, and the half-life of the corresponding pharmaceutically active peptide sequence (X) in the "Hydrolysis in enzyme solution test", is at least 2, preferably at least 3, such as at least 5, more preferably at least 7, such as at least 9, e.g. at least 10, when using the enzyme α-chymotrypsin.

25. A peptide conjugate according to any of the preceding claims, wherein the ratio between the half-life of said peptide conjugate in the "Hydrolysis in enzyme solution test", as defined herein, and the half-life of the corresponding pharmaceutically active peptide sequence (X) in the "Hydrolysis in enzyme solution test", is at least 2, preferably at least 3, such as at least 5, more preferably at least 7, such as at least 9, e.g. at least 10, when using the enzyme pepsin A.

26. A peptide conjugate according to any of the preceding claims, wherein said peptide conjugate has a half-life in human serum or human plasma of at least 10 minutes, such as at least 15 min, e.g. at least 0.5 h, preferably at least 1 h, such as at least 2 h, e.g. at least 3 h, more preferably at least 4 h, such as at least 5 h, e.g. at least 6 h, in particular at least 12 h, such as at least 24 h, e.g. at least 36 h.

27. A method for the preparation of a pharmaceutically active peptide conjugate (X-Z) as defined in claim 2, comprising the steps of:

a) coupling an N-α-protected amino acid in the carboxyl activated form, or an N-α-protected dipeptide in the C-terminal activated form to an immobilised peptide probe H-Z-SSM, thereby forming an immobilised N-α-protected peptide fragment,

b) removing the N-α-protecting group, thereby forming an immobilised peptide fragment having an unprotected N-terminal end,

c) coupling an additional N-α-protected amino acid in the carboxyl activated form, or an additional N-α-protected dipeptide in the C-terminal activated form to the N-terminal end of the immobilised peptide fragment, and

repeating the removal/coupling step procedure in step b) and c) until the desired peptide sequence X is obtained, and then

d) cleaving off the peptide conjugate from the solid support material.

28. A method for the preparation of a pharmaceutically active peptide conjugate (Z-X) as defined in claim 3, comprising the steps of:

a) coupling an N-α-protected amino acid, or an N-α-protected dipeptide to a solid support material (SSM), thereby forming an immobilised N-α-protected amino acid, or an immobilised N-α-protected dipeptide fragment,

b) removing the N-α-protecting group, thereby forming an immobilised amino acid or peptide fragment having an unprotected N-terminal end,

c) coupling an additional N-α-protected amino acid in the carboxyl activated form, or an additional N-α-protected dipeptide in the C-terminal activated form to the N-terminal end of the immobilised amino acid or peptide fragment, and

repeating the removal/coupling step procedure in step b) and c) until the desired peptide sequence X is obtained,

d) coupling an additional N-α-protected amino acid in the carboxyl activated form, or an additional N-α-protected dipeptide in the C-terminal activated form to the N-terminal end of the immobilised peptide fragment, and

repeating the removal/coupling step procedure in step b) and d) until the desired peptide sequence Z is obtained, and then

e) cleaving off the peptide conjugate from the solid support material.

29. A method for the preparation of a pharmaceutically active peptide conjugate (Z-X-Z) as defined in claim 4, comprising the steps of:

a) coupling an N-α-protected amino acid in the carboxyl activated form, or an N-α-protected dipeptide in the C-terminal activated form to an immobilised peptide probe H-Z-SSM, thereby forming an immobilised N-α-protected peptide fragment,

b) removing the N-α-protecting group, thereby forming an immobilised peptide fragment having an unprotected N-terminal end,

c) coupling an additional N-α-protected amino acid in the carboxyl activated form, or an additional N-α-protected dipeptide in the C-terminal activated form to the N-terminal end of the immobilised peptide fragment, and

repeating the removal/coupling step procedure in step b) and c) until the desired peptide sequence X is obtained, and then

d) coupling an additional N-α-protected amino acid in the carboxyl activated form, or an additional N-α-protected dipeptide in the C-terminal activated form to the N-terminal end of the immobilised peptide fragment, and

repeating the removal/coupling step procedure in step b) and d) until the desired peptide sequence Z is obtained, and then

e) cleaving off the peptide conjugate from the solid support material.

30. A method according to any of claims 27-29, wherein the N-α-protecting group is selected from tert.butyloxycarbonyl and 9-fluorenylmethyloxycarbonyl.

31. A pharmaceutical composition comprising a pharmaceutically active peptide conjugate as defined in any of the claims 1-26, and a pharmaceutical acceptable carrier.

32. A pharmaceutical composition comprising
H-Trp-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu-$(Lys-Glu)_3$-OH,
H-Trp-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu-$(Glu)_6$-OH,
H-Tyr-Gly-Gly-Phe-Leu-$(Glu)_6$-OH or
H-Tyr-Gly-Gly-Phe-Leu-$(Lys)_6$-OH, and a pharmaceutical acceptable carrier.

33. A pharmaceutically active peptide conjugate as defined in any of claims 1-26 for use in therapy.

34. A pharmaceutically active peptide conjugate selected from the group consisting of
H-Trp-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu-$(Lys-Glu)_3$-OH,
H-Trp-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu-$(Glu)_6$-OH,
H-Tyr-Gly-Gly-Phe-Leu-$(Glu)_6$-OH and
H-Tyr-Gly-Gly-Phe-Leu-$(Lys)_6$-OH,
for use in therapy.

35. Use of a pharmaceutically active peptide conjugate (X-Z, Z-X, or Z-X-Z) as defined in any of claims 1-26 for the manufacture of a pharmaceutical composition for the treatment or prophylaxis of a condition or disorder, where the peptide sequence X, when not bound to Z, is able to interact with a receptor (or a receptor system) involved with the condition or disorder in question, and where the interaction between X, when not bound to Z, and the receptor (or receptor system) has a therapeutic or prophylactic effect on the condition or disorder.

36. Use of a pharmaceutically active peptide conjugate as defined in any of claims 1-26 for the manufacture of a pharmaceutical composition for use in treatment of pain, HIV, cancer, diabetes, incontinence, hypertension, amnesia, Alzheimer's disease, fever, depression, sex hormone regulation, eating, schizophrenia, osteoporosis and insomnia.

37. A pharmaceutically active peptide conjugate as defined in any of claims 1-26 obtained by means of recombinant DNA-technology.

38. Use of a stabilising peptide probe (Z) of 2-20 amino acid units for the preparation of a pharmaceutically active peptide conjugate as defined in any of claims 1-26,

each amino acid unit in said stabilising peptide probe Z being independently selected from the group consisting of Ala, Leu, Ser, Thr, Tyr, Asn, Gln, Asp, Glu, Lys, Arg, His, Met, Orn, or amino acid units of the general formula I

$$-NH-C(R^1)(R^2)-C(=O)- \qquad (I)$$

wherein R^1 and R^2 independently are selected from hydrogen, C_{1-6}-alkyl, phenyl, and phenyl-methyl, wherein C_{1-6}-alkyl is optionally substituted with from one to three substituents selected from halogen, hydroxy, amino, cyano, nitro, sulfono, and carboxy, and phenyl and phenyl-methyl is optionally substituted with from one to three substituents selected from C_{1-6}-alkyl, C_{2-6}-alkenyl, halogen, hydroxy, amino, cyano, nitro, sulfono, and carboxy, or R^1 and R^2 together with the carbon atom to which they are bound form a cyclopentyl, cyclohexyl, or cycloheptyl ring;

or a salt thereof.